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Feature Papers in Horticulturae II

Edited by

Douglas D. Archbold

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Contents

Preface to "Feature Papers in Horticulturae II"

The goal of this SI is to highlight, through selected works published in Horticulturae in 2021, frontier research in basic to applied horticulture. Some of the articles in this issue focus on species which have not been widely studied. Other papers apply recently-developed techniques to widely-studied species or problems. The reviews selected for this SI summarize recent findings on important research topics and point the way to future research efforts for the wider horticultural community.

Douglas D. Archbold
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Editorial

Feature Papers in *Horticulturae*

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The presented Feature Papers reflect the diversity of the types of research performed on horticultural plant species, spanning from the basic to the applied, production systems, and postharvest studies, in addition to highlighting some critical issues facing horticultural plant species.

The conservation, propagation, and maintenance of wild progenitor species of valuable horticultural plants, as well as species with potential economic and/or environmental value, are facing significant challenges due to climate change, urban encroachment into undeveloped areas, and invasive species. Marler et al. [1] reviewed the biology, ecology, horticulture, and conservation of the rare, endangered leguminous tree species *Serianthes nelsonii* Merr. As tree mortality is a critical problem, techniques for the successful propagation of the species by seed and via air-layering were discussed as examples of solutions to building seedling populations. However, issues with in situ seedling mortality following transplantation and during long-term growth are continuing concerns which future research must address.

Invasive pest species can have significant negative impacts on plant populations. Marler et al. [2] discussed the critical problem of the armored scale (*Aulacaspis yasumatsui*) to cycads (*Cycas revoluta* Thunb) internationally. They covered the biology, invasion chronology, and host-plant responses of this pest species and efforts in its control. International movement of nursery plants has enabled this pest to negatively impact the international cycad horticulture trade, and growth of the scale population may remain unchecked until efforts establishing non-native biological control can be developed and implemented.

Ornamental species may contribute to improved slope stability along urban and suburban areas. Francini et al. [3] indicated that if ornamental species are to have desirable effects on slope stability, they must have good tolerance to abiotic stresses, such as high and low temperatures, drought, pollution, and nutrient deficiency. The plants that can be used for reducing the erosion of slopes must be in full growth during periods with a higher incidence of rain, and must also be compatible with the temperature ranges of different seasons. Root growth may be considered as a key factor in their usefulness, and could be a useful criterion upon which to select the species best suited for such situations.

The caper (*Capparis spinosa* L.) is currently considered as at risk of genetic erosion, mainly due to overgrazing and overharvesting. This situation may be made more serious because of the lack of efficient propagation techniques, with the caper commonly considered a "difficult-to-propagate species". Sottile et al. [4] reviewed the main available sexual and vegetative propagation techniques with the aim of assessing whether, and to what extent, this characterization is correct. While seeds show a physiological dormancy that can be lowered by hormonal treatments, vegetative propagation by in vitro techniques appears quite effective. Thus, propagation of the caper is not limiting dissemination of the species, and caper plant material should not be limited for cultivation in high-density, intensive plantings.

Understanding the symbiotic plant–fungus interaction of the orchid *Cattleya purpurata* is valuable for the propagation of this valuable species. Bazzicalupo et al. [5] studied the seed micromorphology, in vitro germination, and early stage seedling morphological traits of the species. Seed morphology was comparable to that of other congeneric species, showing classical adaptations related to aerodynamic properties and to seed wettability.

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Seedling morphology was found to be in line with other taxa from the same genus, showing characteristics typical of epiphytic species.

Production systems for horticultural crops are constantly evolving as new strategies for planting as well as disease and pest management emerge. This is especially true for protected systems. Nicola et al. [6] assessed a closed recirculating system applicable to both open fields and protected cultivation. A lab-scale pilot plant was designed for a standardized and reproducible growing system and was evaluated with production trials of head and multileaf vegetable species and culinary herbs at high plant densities. Both culinary herbs and leafy vegetables performed well in the system. Thus, such a standardized system may be useful for testing numerous other species, substrates, hydroponic nutrient solutions, and fertigation scheduling.

Grilo et al. [7] reported that olive (*Olea europaea*) tree planting density in hedgerows and canopy position affected "Cerasuola" and "Koroneiki" olive oil quality. Fruit in the upper layers of the canopy consistently showed a higher maturity index, higher fat content, higher phenol content and lower water content than lower layers. However, high-density trees showed the largest differences in fruit maturation and water and fat content between upper and lower canopy positions, increasing quality and oil yield variability at harvest. Thus, cultivar, planting density, and canopy architecture may be strong determinants of olive oil yield and composition in hedgerow planting systems.

Zucchini et al. [8] determined whether there is a daily growth hysteresis versus vapor pressure deficit in cherry (*Prunus cerasus*) fruit. Their results indicated that hysteresis can be employed to evaluate the initial phenological phase of fruit maturation, as a fully clockwise hysteresis curve was observable only in the fourth stage of fruit growth. They also discussed how there are opportunities for its use in the management of fruit production, such as for irrigation timing in precision fruit farming.

To improve the understanding of bud development in low- and high-chill Japanese apricot (*Prunus mume*) genotypes, Hsiang et al. [9] characterized floral bud development using a modified BBCH scale and analyzed the relationship between BBCH stages and floral primordium development and the dormancy phase transition in cultivars of each chill type. The floral bud developmental period corresponding to BBCH stages 51–53 includes the transition from endodormancy to ecodormancy. Male meiosis and microspore development occurred during this transition in high-chill cultivars but were detected considerably later than the transition in a low-chill cultivar. A slow or suspended developmental phase was observed only for the high-chill cultivars upon completion of floral primordium organ differentiation, suggesting that chilling may be required to induce floral bud maturation and dormancy release only in high-chill cultivars. Possible relationships among BBCH stages, flowering-related morphological characteristics, and the dormancy phase transition in Japanese apricot are discussed.

New horticultural crops are introduced with regularity to address niche markets. As an example, a growing market for sweet and colorful mini peppers (*Capsicum annum* L.) has been developing, and Giacomin et al. [10] studied postharvest quality and sensory evaluation of the mini sweet peppers. Physical–chemical, nutritional, and sensory analyses of several genotypes indicated that total carotenoids, phenolics, antioxidant activity, vitamin C content, fruit firmness, and sensory analysis of some genotypes were superior to others and may be most desirable to consumers.

Disease and pest management techniques of horticultural crops have been undergoing major changes in recent years, with the push to create more sustainable production systems. While published studies on the methodologies for evaluating stone fruit susceptibility to brown rot are abundant, Mustafa et al. [11] reported that significant variation in the various approaches have limited the ability to compare results from different studies. Thus, they reviewed the literature on phenotyping brown-rot susceptibility in stone fruit, focusing on peach (*Prunus persica*), and discussed ways to manage major factors affecting brown-rot phenotyping studies. Experimental results from multiyear evaluation trials are also described, highlighting year-to-year variability and exploring correlations of

evaluation outcomes among years and assay types, suggesting that choice of phenotyping methodology must be carefully considered in breeding programs.

Marques et al. [12] described advances and challenges in the use of RNA interference (RNAi) technology for citrus Huanglongbing vector control. Despite the availability of specific silencing sequences aimed at a target gene of the insect pest vectors, the uptake of double-stranded RNA is limited in hemipteran insects. Thus, improved delivery methods, stability maintenance, and RNAi response are primary factors contributing to increased effectiveness of exogenous RNAi against hemipteran pests. These approaches can serve as potential tools for efficient disease control.

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Review

Adaptive Management Lessons for *Serianthes nelsonii* Conservation

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Abstract: The literature covering the biology, ecology, horticulture, and conservation of the critically endangered tree *Serianthes nelsonii* Merr. was reviewed. The roots, stems, and leaves of this charismatic legume tree revealed highly plastic traits and responded positively to horticultural manipulations to improve the quality of container-grown transplants. Pre-sowing seed treatments of seed coat scarification and 1 h of imbibition generated 85% to 90% germination at a temperature optimum of 26 °C. Adventitious root formation on air layers and successful unions on approach grafts were 100%. Seedling and sapling growth was maximum under 25% to 50% sunlight transmission, limited irrigation to ensure adequate root zone aeration, repetitive stem tip pruning to increase root:shoot quotient, and thigmic stress to retain an orthotropic orientation of stems. In situ regeneration on Guam was substantial but recruitment from seedling to sapling was nil. High quality leaf litter chemistry enabled rapid decomposition, and soils beneath the tree exhibited unique chemical traits that increased ecosystem health by creating spatial heterogeneity. The greatest unanswered questions focus on plant mortality. Research is needed to determine the reasons for the mortality of in situ seedlings, mortality within transplantation projects on Guam, and the mortality of 60% of the mature in situ tree population during the 26-year implementation of the national recovery plan. Horticultural researchers are ideally positioned to answer these urgent questions.

Keywords: adaptive management; conservation science; Guam; Mariana Islands; Rota

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1. Background

The endemic *Serianthes nelsonii* tree was described in 1919 [1] when the global population spanning the adjacent Mariana Islands of Rota and Guam was already limited in size. This attractive legume tree was added to the United States Endangered Species Act (ESA) when it was listed as endangered in 1987 [2]. The original 1978 endangered assessment by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species was upgraded to critically endangered in 1998, and this designation was confirmed in a 2016 assessment [3]. A national species recovery plan was published in 1994 when the global population was reported as 122 trees [4].

Tree heights of 36 m and bole diameters of up to 2 m [1,4] indicate that *S. nelsonii* is one of the largest native trees within its endemic range. The bi-pinnately compound leaves are comprised of small leaflets up to 5 mm in length [1,4], providing the tree canopy with a distinctive fine texture as a landscape specimen. The green to white calyx and corolla are not showy, but the numerous stamens are striking with red to maroon filaments capped by yellow anthers [1,4]. As a legume tree that associates with nitrogen-fixing endosymbionts [5], its presence infuses the biosphere's nitrogen into the terrestrial food web.

Guam's single mature *S. nelsonii* tree is located within one of the expansive military bases. The burgeoning military construction activities have destroyed much of the forest, creating an exclusive case study that has been discussed within the context of indigenous

peoples' rights [6–8]. This tree has emerged as a symbol of the inability of indigenous CHamoru peoples to have an adequate voice concerning the ecocide of the natural systems that define their heritage and culture [9,10]. These unique facets of conserving this endangered charismatic tree indicate the stakeholders deserve for military decision-makers to conduct all conservation efforts with transparency and the best available knowledge.

The national species recovery plan [4] included the need for research to more fully understand the conservation requirements for species recovery. United States recovery permits are issued to support research activities to expand knowledge for conserving ESA-listed species [11]. Two permits have been issued for the recovery of *S. nelsonii*. A literature review of the resulting published research has not been conducted to date. Herein, we have updated the history of published information by conducting a systematic literature review. First, we reviewed the historical *S. nelsonii* publications and then added recent adaptive management publications, focusing exclusively on peer-reviewed journal papers. Second, we used the literature and the reported progress on the species recovery efforts to list recommendations that may improve impending species recovery efforts. These recommendations may have application to other case studies of endangered tree species comprised of extremely small global populations that have not been conserved successfully despite long-standing formal conservation programs.

2. Review Methods

We conducted a Google Scholar search with "Serianthes nelsonii" as the only query term. Our review was restricted to peer-reviewed publications that advanced the national recovery plan mandate of relevant research [4], and the search results were so constrained that we did not need to refine or filter the query terms. This approach ensures our methods are reproducible, that review results can be updated as necessary, and that we avoided the mistakes made when gray literature and anecdotes are used in conservation decisions [12].

3. Historical Literature Established the Foundation

The first *S. nelsonii* paper that was not taxonomic in nature was a 1995 description of general field observations [13]. This paper communicated that the major threats to the species were tropical cyclones, ungulate herbivory, mealy bug herbivory, and damage from the butterfly *Eurema blanda* Boisduval.

This review found only one historical peer-reviewed article that was informative, and this paper communicated results of field and nursery observations from 1983–1993 [14]. The 1994 national recovery plan was based on the global population of the 122 trees constricted to Guam and Rota that was described here. This included 121 trees on Rota and one tree on Guam, with descriptions of documented mortality of trees that were reported in the 1970s and 1980s. Seed production was reported as abundant, with as many as 1000 fruits observed in a single tree and fruits containing a mean of five seeds. Despite the copious seed production, regeneration was identified as a major bottleneck, as very few seedlings were observed during the field work. Damage from wild deer (*Rusa marianna* Desmarest) and feral pigs (*Sus scrofa* L.) was reported as a major threat to *S. nelsonii*. Herbivory from the butterfly *E. blanda* was reported as a threat on Guam, but not on Rota. Numerous mealy bug species were collected from *S. nelsonii* plants, with *Dysmicoccus neobrevipes* Beardsley, *Dysmicoccus brevipes* Coccerell, and *Planococcus citri* Risso causing the greatest damage. To survive in the Mariana Islands, native tree species must resist damage during frequent tropical cyclones; however, *S. nelsonii* trees suffered mortality events in the 1988, 1990, and 1992 tropical cyclone seasons. Most tree loss due to habitat conversion occurred prior to written records of *S. nelsonii* population developments, though one tree was reportedly killed during military construction in the 1970s. Inbreeding and wildfires were also discussed as possible threats to species recovery.

4. Management Can Be Based on Sound Science

4.1. Nursery Production Not a Limitation

Serianthes nelsonii seeds utilize physical dormancy mechanisms, and several studies were conducted to improve pre-sowing and germination treatment protocols. An anecdotal description of *S. nelsonii* seed treatments was published in 1997 and indicated that clipping the seed coat prior to imbibition was an effective pre-sowing seed treatment [15]. The observational study did not include experimental design or statistical approaches employed. Sandpaper was used to scratch through the seed coat until cotyledon tissue was visible, then the seeds were imbibed in municipal water for up to 24 h [16]. Germination was insufficient with these long periods of imbibition. Therefore, the periods of imbibition were shortened to determine 1–2 h of imbibition was successful.

Seedling emergence and seedling growth studies under a range of sunlight transmission from 25% to 100% indicated the greatest level of shade led to the greatest seedling emergence percentage of 85% [16]. Similarly, the emergence velocity under 25% sunlight transmission was more than triple that under 100% sunlight transmission. The full sun treatment killed 100% of the seedlings shortly after emergence, and 12 weeks of height growth of the remaining seedlings was greatest in 25% sunlight transmission. This study also confirmed that *S. nelsonii* seeds are desiccation-tolerant and can be stored with no decline in germination capacity for 9 months [16].

Pre-sowing seed treatments and germination protocols were studied to develop a highly successful horticultural program. Scarified and non-scarified seeds were imbibed in aerated water for 24 h to determine that a minimal increase in fresh weight occurred during the initial 1 h for *S. nelsonii* seeds without scarification [17]. No further increase in fresh weight occurred. In contrast, a substantial increase in fresh weight occurred over the entire 24 h period of imbibition for scarified seeds [17]. Seed respiration increased within 3 h of imbibition and continued to increase in a linear pattern until germination. The scarified, imbibed seeds initiated germination under darkness within 50 h and completed germination within 60 h.

The predicted optimum temperature for *S. nelsonii* germination was 26 °C within a range of treatments from 14 °C to 39 °C [17]. High temperature inhibition of germination above the optimum was greater than low temperature inhibition below the optimum. These results indicated that the inhibition of seedling emergence and early growth under full sun [16] may be caused by indirect heat stress rather than by direct light stress.

Imbibing *S. nelsonii* seeds with gibberellic acid solution (range of 0–300 $mg \cdot L^{-1}$) or nitrate solution (range of 0–3000 $mg \cdot L^{-1}$) did not influence germination percentage or speed [18]. These chemical priming treatments did increase hypocotyl length, cotyledon longevity, and initial seedling growth, but the growth responses were ephemeral and the treatments did not influence the ultimate time required to each 30-cm in plant height. Scarified, imbibed seeds incubated in high red:far red light or low red:far red light behaved similarly, indicating *S. nelsonii* seed germination was not influenced by incident light quality [18].

These initial studies were used to design a production trial to determine the speed that a qualified nursery team could grow *S. nelsonii* plants for transplantation to in situ recovery plantings. The germination of seeds and the initial growth of seedlings were nurtured under rain protection and 25% sunlight transmission for two months, and this was followed by long-term seedling management with minimal irrigation frequency under 50% sunlight transmission. These methods generated 1 m tall plants in 8 months and 2 m tall plants in 12–13 months [19]. The horticultural skills and knowledge of water relations that are required to achieve this growth are discussed hereinafter.

Studies of asexual propagation in *S. nelsonii* revealed highly successful outcomes [19]. Traditional air-layer techniques installed on lateral stems of 90–130 cm tall plants using 3 $mg \cdot g^{-1}$ indole-3-butyric acid administered in powder form and sphagnum moss as the rooting medium led to 100% success in adventitious root formation within 12 weeks. Half of the air layer propagules declined and died shortly after excision from the source plant,

but the remainder developed into healthy plants with adventitious roots. Approach graft techniques were employed to determine that the use of *Serianthes kanehirae* Fosberg rootstocks led to 100% success [19]. This same rootstock was used to show that an experienced horticulturist could achieve a 25% success rate using veneer graft techniques. These studies revealed the utility of asexual propagation to augment sexual propagation as a means of increasing the number of plants available to meet the goals for species recovery.

Water management is equal in importance to shade management in a *S. nelsonii* nursery. This propagation study also discussed refinements of these factors [19]. Because containerized seedling growth is constrained by Guam's copious rainfall, excluding rainfall from the container media for at least eight weeks maximized seedling growth. Rainfall exclusion is also important after every occurrence that temporarily reduces the leaf:root quotient of the nursery plants, such as insect defoliation or stem pruning. This was explicitly studied by using a fixed irrigation schedule versus a tensiometer-controlled irrigation schedule following a pruning operation [19]. For several weeks after pruning, irrigation events for plants with irrigation based on matric potential were separated by more than two weeks. These plants initiated regrowth rapidly. In contrast, the plants that continued to receive the pre-pruning irrigation schedule every 3 days were stunted. All plants appeared healthy in appearance, but the height increment for the over-watered plants was 20% of plants that received irrigation based on matric potential. One caveat to these interpretations is that the studies were conducted in the standard container medium of 60% peat and 40% perlite, and that the use of a more aerated container medium may be an alternative means of increasing plant growth even if over-watering occurs. The restrictions imposed by the federal handling permits do not allow the use of many locally available substrates that could be used to improve drainage and aeration in container nurseries.

These adaptive management studies with container-grown *S. nelsonii* seedlings collectively verify that this species is highly intolerant of over-watering by fixed irrigation systems as well as exposure to rainfall. Rapid growth can be achieved by monitoring symptoms of over- and under-watering through daily inspection of individual plants. The plants require long durations to recover from over-watering mistakes, but are highly resilient following mild drought stress after inadvertent under-watering.

These studies [16–19] were designed to determine the reasons behind the published assertions that *S. nelsonii* is difficult to grow in a managed nursery [4,14]. The findings did not confirm these assertions, but instead verified that this endangered tree is among the easiest of plant species to grow in a container nursery. Research horticulturists are ideally equipped to conduct plant adaptive management research [20], and this case study is an example that confirms this assertion.

4.2. In Situ Regeneration Not a Limitation

Twice monthly visits to the single surviving *S. nelsonii* tree in northern Guam were used to record every emerged seedling for the full span of 2013, and visits were continued until 100% seedling mortality had occurred [21]. The emergence of 374 new seedlings beneath this single tree represented more than one new seedling per day for the year. However, about 30% of the seedlings died in less than 15 days, only 10% lived longer than three months, and every seedling died when the study was terminated in March 2014. The dry season for Guam is generally January to June, and the rainy season is generally July to December. However, the transition months are not clearly demarcated each year. A strong seasonal pattern was evident with the least number of seedlings emerging in the second half of the dry season and the greatest number of seedlings emerging in the first half of the rainy season. Seasonal aspects of seedling longevity also verified greater mean longevity during the rainy season, indicating that drought stress of the small seedlings with limited root systems may be one factor responsible for rapid *S. nelsonii* seedling mortality. This study revealed for the first time that regeneration was substantial, that seedling mortality was rapid, and that the major limitation to the natural expansion of the plant population was a failure of seedlings to recruit into the sapling stage.

These methods were repeated using weekly visits to the tree from October 2014 until October 2015 [22]. Of the 243 seedlings that emerged during the 12-month period, 30% died in less than two weeks, corroborating the results of the first study [21]. The weekly visits confirmed that about half of these seedlings died in less than 7 days. This study revealed that increased wind events also profoundly influenced *S. nelsonii* regeneration dynamics. Litterfall traps were used to determine the seasonal aspects of seed rain. The results indicated that a single wind event generated 46% of the annual seed rain. A tropical cyclone damaged northern Guam forests on 15 May 2015, leading to an abrupt increase in seedling emergence rates. Indeed, more than 17% of the annual seedling emergence events occurred in the four weeks following this tropical cyclone. The two-week mortality count abruptly declined during the months following the tropical cyclone, verifying greater longevity.

Two of the historical claims concerning *S. nelsonii* conservation have been clarified by these field methods. First, the claim that *S. nelsonii* regeneration is a major conservation limitation [4,14] was developed from ad hoc observations rather than from a sustained schedule of visits. This is an example of the need for repetitive observations to avoid misinterpretations of the reasons for declines in populations of threatened tree species on islands [23]. Regeneration is not a major *S. nelsonii* conservation issue that should be studied until recruitment limitations are further studied and more fully understood. Indeed, the reasons for 100% recruitment failure as a result of rapid seedling mortality should be the primary focus of future ecology research. Hundreds of *S. nelsonii* nursery plants have been grown and transplanted to in situ or circa situ sites since the 1990s, and post-transplant mortality following these expensive conservation projects further validates the need to study recruitment. We believe some of the factors that are causal of in situ seedling mortality are also causal of transplant mortality following the removal of the plants from conservation nurseries. Second, the threats illuminated in 1995 as tropical cyclones, ungulate herbivory, and insect herbivory [13] were not causes for the very rapid seedling mortality that was documented. The seedling observations occurred within a functioning ungulate exclusion fence. The single tropical cyclone that occurred during the 2015 field work was not detrimental, but instead provided beneficial outcomes for subsequent seedling emergence and longevity. The weekly schedule of site observations never illuminated chronic infestations of insect herbivores on newly emerged seedlings. These two years of frequent seedling observations [21,22] revealed that the factors responsible for the lack of recruitment are not readily apparent, and research to determine these factors remains a high priority for the conservation of the tree species.

4.3. The Leaf

The *S. nelsonii* leaf is a bi-pinnate compound organ with numerous small leaflets [1]. Highly active pulvini are positioned at the petiolule of each leaflet, and the resulting leaflet movement behavior has been quantified [24]. Full sun leaves initiated abrupt paraheliotropic movements by 09:00 h on sunny days and reached a maximum angle above the horizontal of 80° by midday. Shaded leaves began paraheliotropic movement later in the morning and the extent of movement was muted, especially in deep shade of 22% sunlight transmission. These movement patterns elicited several outcomes, some of which were clearly beneficial mechanisms for avoiding high light stress. For example, leaflet temperature was 8 °C above ambient when full sun leaflet paraheliotropism was disallowed, but only 4 °C for leaflets that were allowed to move naturally. Similarly, quantum efficiency of photosystem II declined to about 0.25 during midday when full sun leaflet paraheliotropism was disallowed, but remained above 0.55 for leaflets that were allowed to move naturally. These data reveal that paraheliotropism of *S. nelsonii* leaflets enables the protection of the photosynthetic machinery during high light exposure. An interesting observation that deserves more research was that nyctinastic nocturnal leaflet movements were similar in amplitude to diurnal paraheliotropic movements, with full

sun leaflets moving at night to a vertical orientation and the shaded leaflets moving to a maximum of about 50° above horizontal.

The influences of incident light during *S. nelsonii* leaf construction on leaf morphology and leaflet anatomy were determined for a range of incident light from 6% to full sun conditions [25]. The laminae thickness exhibited acclimation with upper epidermis, palisade mesophyll, spongy mesophyll, and lower epidermis thickness reduced in deep shade and increased in full sun. The upper epidermis and palisade mesophyll layers exhibited the greatest changes in thickness. Other leaflet traits such as total area and leaf mass per area were also highly dependent on incident light, with leaflet area greater in shade and leaf mass per area greater in sun. Whole leaf traits were also highly responsive to incident light during leaf construction. For example, longer petioles and a wider insertion angle between the rachillae and rachis occurred in shade leaves, which enabled the leaflets to be displayed over a much larger plagiotropic two-dimensional area to capture more incident light in the shaded conditions.

The research to date indicates that the *S. nelsonii* leaf is a highly responsive organ that can modify anatomy, morphology, and behavior to best exploit the prevailing light conditions. The considerable acclimation potential that has been shown for this bi-pinnate compound leaf confirms that *S. nelsonii* is representative of late successional species with seedlings with leaves that must contend with the shade of the forest understory and adults with leaves that occupy parts of the emergent forest canopy [26–28].

A full understanding of leaf traits for tree species that are native to the Mariana Islands includes where they fit in the leaf economics spectrum [29] and the approach for how each species responds to the threats imposed by tropical cyclones [30]. One end of the spectrum is characterized by tree species that produce expensive leaves that maintain mechanical integrity with long lifespan, and these species resist tropical cyclone damage by constructing strong stems that can withstand the wind forces despite extensive wind drag of the canopy. The opposing end of the spectrum is characterized by tree species that produce inexpensive leaves that are dislodged from the stems during a tropical cyclone, and this saves the trees from structural damage because the wind drag is minimized by the absence of leaves. An example of this is *S. nelsonii*. For *S. nelsonii* and other species with inexpensive leaves, the re-construction of new leaves following a defoliating tropical cyclone is rapid (Figure 1a).

(**a**) (**b**)

Figure 1. Vegetative organs of *Serianthes nelsonii* exhibit rapid growth rates. (**a**) One month after complete defoliation during a 15 May 2015 tropical cyclone, the emergent canopy of a mature tree exhibited fully expanded healthy leaves and flowers; (**b**) A five-month-old nursery plant exhibited rapid stem growth and robust leaves due to shade and under-watering in the nursery. The rapid stem growth produced weak stems that were unable to maintain orthotropic orientation, inducing lateral bud growth that reduced plant quality.

4.4. The Structural Organs

Stems and roots provide crucial functions for plant survival, including the structural traits that improve competitive advantages. For late successional species, this includes stem growth that eventually positions leaves in the upper strata of the forest canopy. The

greatest height increment for young *S. nelsonii* plants occurred in shade of 25% sunlight transmission [16]. However, inducing these magnitudes of height growth rates reduced the tree's out-planting success and required horticultural treatments to produce high quality transplants.

First, *S. nelsonii* saplings that are grown rapidly in a shaded nursery are prone to bending over due to biomechanically weak stems [31]. Therefore, the upper canopy stems of these saplings do not maintain an orthotropic orientation. The stem lean removes apical dominance and induces lateral bud break and growth that creates an undesirable shrubby nursery plant (Figure 1b). Twice daily imposition of stem flexure revealed that *S. nelsonii* stems exhibited thigmomorphic responses that increased stem strength [31]. This simple horticultural procedure maintained the desired orthotropic orientation of the treated stems. The difference in stem angle of control plants versus treated plants was increased by an experimental wind load at the end of the study. Force displacement curves revealed the force required to bend the treated stems was increased more than 60% above that of the control plants. The results indicated that shade and some form of thigmic stress to stems may be combined as horticultural protocols to obtain the height growth advantage of shaded conditions without the disadvantage of weak stems.

Second, *S. nelsonii* saplings that are grown rapidly under shade in a container nursery do not develop adequate root systems to sustain plant health and viability after transplantation to a field site [32]. Repetitive heading back pruning of the stem leader to temporarily stop stem extension may be one method that improves the relative root growth of container-grown plants [32]. Because destructive techniques are needed to unambiguously quantify root growth, *S. kanehirae* and *Serianthes grandiflora* Benth. were employed as surrogate congeneric species to study this phenomenon. This simple horticultural protocol generated beneficial increases in the root:shoot quotient of 56% for the pruned plants compared with the control plants. The plants were transplanted to a closed forest site with soils that characterize the areas of occupancy of *S. nelsonii*, and after one year of growth 70% to 80% of the control plants were dead and 100% of the pruned plants were alive. The control plants that remained alive after one year exhibited stem die-back and constrained height increment, but the plants that were repetitively pruned in the nursery exhibited no stem die-back after transplantation. Following one year of growth, the pruned plants were 28% to 41% taller than the plants that were not pruned in the nursery.

These studies suggest that much of the *S. nelsonii* plant mortality following transplantation from conservation nurseries since the 1990s has been caused by the limited root system of the plants produced in shaded container nursery conditions. Practitioner qualifications and the proficiency of nursery management should therefore be determined by the survival and height of the transplanted stock one or more years after removal from the nursery, and not solely by the number of transplants produced and their appearance after leaving the nursery. The ultimate mortality of transplanted *S. nelsonii* plants demonstrates the necessity of nursery managers' use of repetitive stem pruning or some other treatment that increases the root:shoot quotient of the transplants.

There is substantial evidence that practitioners also produce transplants that are destined to fail by over-watering containerized *S. nelsonii* plants. The literature on hypoxia and anaerobiosis, e.g., [33–35], can be used to understand the root damage that is caused when practitioners make this mistake. Root death, secondary attack of root pathogens, stunted stem growth, and leaf epinasty mistaken for wilting are among the cascade of plant responses that occur when practitioners without an understanding of plant water relations over-water *Serianthes* plants in containers. Understanding the root growth traits of this species is key to cultivating successful transplants.

The diel pattern of root extension was determined for *S. grandiflora*, *S. kanehirae*, and *S. nelsonii* for small seedlings to 2-m tall saplings [36]. The percentage of daily root extension that occurred during the nocturnal period was 58% to 72% for small seedlings, and this decreased to 51% to 55% for 2-m tall saplings. Root extension rates of about 1 cm per day for *S. nelsonii* to 2 cm per day for the other species were greater than corresponding stem

extension rates. These data may be used to visualize a theoretical *S. nelsonii* root span of a 12-month-old in situ sapling. The theoretical 12-month-old in situ sapling would be supported by a 365-cm radius root system. In contrast, a nursery transplant of the same age would begin the post-transplant recovery period with a root system radius that is half the diameter of the container. This theoretical exercise illuminates the competitive disadvantage that a container-grown *S. nelsonii* sapling experiences immediately after being transplanted into competitive in situ settings.

An understanding of root egress behaviors of woody plants following removal from a container nursery is needed to determine the most appropriate nursery protocols. Air-pruning and chemical-pruning (copper hydroxide) techniques were employed to grow *S. nelsonii* plants in rigid containers, and root extension growth after transplantation was quantified using rhizotron windows [37]. Lateral root egress was greatly accelerated by the root pruning container treatments compared with the control containers. More importantly, the proportion of lateral root egress near the soil surface was greatly increased by root pruning containers, with most of the root egress from control containers occurring near the bottom of the containers. Lateral root extension rates of the plants in the root pruning container treatments were increased more than 50% above those of the plants in the control containers.

4.5. Ecology

The reported lack of *S. nelsonii* seedling-to-sapling recruitment [21,22] may be a factor of the concepts described in the Janzen–Connell hypothesis [38,39]. In general terms, this hypothesis predicts reduced seedling growth and survival under conditions of minimal distance from the parent tree and increased density of con-specifics. Preliminary investigations to understand the reasons for the rapid in situ seedling mortality were conducted using paired seedling treatments [40]. The studies controlled for insect herbivory by applying imidacloprid for Hemiptera and carbaryl for Lepidoptera herbivores. Weekly applications of soluble fertilizer mitigated nutrient deficiency stress. Soil-borne pathogens were controlled with mefenoxam fungicide drenches. Low light stress was mitigated with 12-volt lamps powered by a solar system which provided photosynthetic active radiation of 205 μmol $m^{-2} \cdot s^{-1}$. The fungicide and supplemental light treatments lengthened seedling lifespan, but the fertilizer and insecticide treatments were ineffective for extending seedling lifespan. The results indicated that a buildup of root pathogens near the parent tree and the limited light of the forest floor were two of the factors that may combine to cause the ongoing recruitment failures.

Nutrient availability beneath the *S. nelsonii* tree was characterized through the analysis of leaf litter decomposition rates. Rapid litter decomposition was predicted for *S. nelsonii* leaf litter because of low lignin concentrations of only 148 $mg \cdot g^{-1}$ (dry weight) and a carbon/nitrogen quotient of only 23 [5]. These predictions were verified during the use of litterbag techniques to reveal a loss of about 80% of the initial litter carbon and nitrogen after only three months of incubation. The addition of *S. nelsonii* litter to soils (1% based on dry weight) increased the initial soil carbon dioxide efflux 6.5-fold, but efflux converged with that of control soils after only 50 days. These results indicated that *S. nelsonii* litter greatly increased heterotrophic respiration of the decomposer community, but the increase was short-lived because of the high-quality litter. The incubation of one liter of soil after the addition of a 30-g sample of *S. nelsonii* leaf litter revealed that a 400% increase in available nitrogen occurred in 120 days. Under the conditions of this buried bag study, most of this available nitrogen was nitrate. These results indicated that competitive plant acquisition of available nutrients in a forest setting and the rapid leaching of nitrates under Guam's abundant rainfall may remove the available nitrogen from the soils beneath *S. nelsonii* trees.

A paired study designed to compare the chemistry of surface soils beneath a mature *S. nelsonii* tree with soils in adjacent forest cover [41] confirmed the predictions of the incubation studies. Total nitrogen concentration was more than five-fold greater in the soils beneath this legume tree, but available nitrogen concentration was greater in the

nearby soils that were not influenced by the *S. nelsonii* tree. A 133% increase in nitrification in the soils from beneath the *S. nelsonii* tree indicated the copious generation of nitrates from the high-quality litter. As predicted, these labile nitrates were not retained in the soil system in an open forest. Long-term effects of the mature *S. nelsonii* tree on soil nutrient relations were not homogeneous, with potassium, calcium, magnesium, manganese, and iron concentrations existing in greater quantities in the soils away from the *S. nelsonii* tree, but zinc and copper concentrations being greater in the soils beneath the *S. nelsonii* tree. The influence of Fabaceae trees on localized soil nutrients increases neighborhood biodiversity [42]. These results [5,41] illuminated how *S. nelsonii* trees increase ecosystem health by adding newly fixed nitrogen for the soil food web and providing spatial heterogeneity of the soil chemical and biological traits.

4.6. Summary of Horticultural Management Recommendations

The literature prior to the recent adaptive management studies asserted that *S. nelsonii* is difficult to grow in conservation nurseries [4,14]. The outcomes from providing academic scientists an opportunity to establish adaptive management studies revealed the tree species is actually among the easiest of plant species to grow in a container nursery. These studies elucidated four crucial factors that must be understood by practitioners in order to rapidly produce a healthy *S. nelsonii* container-grown transplant. First, water management is crucial. Protection from rainfall and the irrigation of each container individually based on plant phenotype is an effective approach to avoid the growth-constraining mistakes of over-watering. Second, shade of ≈25% sunlight transmission for germination and early seedling growth and ≈50% sunlight transmission for continued sapling growth will enable rapid growth at the seedling-to-sapling stage. Third, repetitive stem tip pruning or some other treatment is required to construct an adequate root system to prevent rapid post-transplant mortality. Protection from rainfall and reduced irrigation frequency is required for several weeks following each pruning event. Fourth, periodic mechanical stimulation of the stem is required to retain an orthotropic stem orientation of a shade-grown sapling, which is crucial for reaching the goals of sapling height as rapidly as possible. A skilled horticulturist with proficiency in plant water relations and an understanding of how light communicates with plants will possess the skills to use the confluence of these four factors to enable the production of 200-cm tall healthy transplants in less than one year (Figure 2).

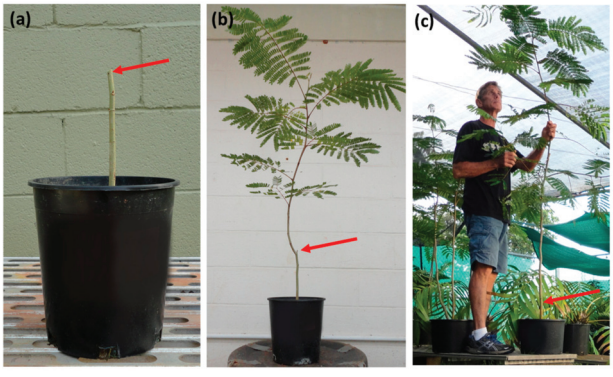

Figure 2. Recovery of *Serianthes* plants from heading back pruning is rapid if a plant physiologist is available to manage the water relations decisions. (**a**) Three-month-old *S. grandiflora* seedling pruned to 15-cm height on 3 August 2015; (**b**) Same plant at 56 cm height on 15 September 2015. (**c**) Same plant at 203 cm height on 26 February 2016. Red arrows point to the position of initial pruning. Three more stem pruning steps were administered for a total of four pruning steps, each followed by restrictive irrigation and protection from rainfall.

5. Research and Conservation Recommendations

The national recovery plan for *S. nelsonii* called for the addition of 2000 mature individuals among four 500-tree sites to augment the existing 122 trees as a first step to down-list from endangered to threatened [4]. The 1994 plan outlined a 16-year agenda to reach this goal. As of 2020, the tree population has declined to 49 trees, and no mature trees have been added to the Guam population despite the long history of funded conservation projects [43]. Regarding the plan for two 500-tree in situ managed populations on Guam, only 17 recently planted saplings represent progress toward this goal [44]. If the successes of out-planting projects since the 1990s are used as predictors, these 17 saplings will die in the near future with no accompanying adaptive management research to illuminate the reasons for the mortality.

These facets of *S. nelsonii* recovery reveal the fact that knowledge needed to inform consequential decisions has not been adequately pursued and remains elusive for most relevant issues. Several crucial recommendations for *S. nelsonii* conservation were discussed in 1996 [14], and the implementation of these recommendations has been inadequate and remains urgent. These recommendations included the study of the tree's breeding system, research to improve propagation protocols, and the determination of the minimum sustainable population size. The addition of recent adaptive management research has enabled several more practical recommendations. Here, we discuss our recommendations to improve species recovery efforts.

5.1. Add Adaptive Management Research to All Funded Projects

Plant species in tropical regions are twice as threatened as plant species in temperate regions due to anthropogenic activities [45]. Plant extinctions occur on islands at a greater pace than in continental habitats due to greater vulnerability to disturbance events [46]. Plant species with extremely small populations are among the most threatened because of the limited geographical distribution and constrained genetic diversity [47]. *Serianthes nelsonii* is the hallmark of these global conservation realities, so developing and implementing an effective conservation and recovery plan must be based on sound science [47,48]. This endemic species population comprised of less than 50 mature trees emerges as an archetypical case study where recommendations for the co-production of new knowledge have not been adequately implemented in historical conservation projects. How can sound science be exploited if the required new knowledge is not generated and published by the funded practitioners?

The minimal success in reversing the extinction threats of *S. nelsonii* has occurred without a sustained effort to develop an understanding of the reasons for the lack of success. The decades of failures to advance toward the goal of 2000 mature trees within four managed sites have unfolded without the involvement of knowledgeable academic scientists in the field work following transplantation from conservation nurseries. The active co-production of new knowledge through adaptive management studies [49–51] will be required to build a substantial foundation of sound science to guide future species recovery. New knowledge is only reliable for conservation decisions when it is embedded in the primary literature because the process is filtered through the peer review procedures which ensure defendable and repeatable adaptive management methods. Indeed, research into any facet of plant biology or conservation is not finished until it is published [52], as this is the safest approach for archiving the findings for future access. The mountain of knowledge that is available in the primary literature is the foundation that ensures future research avoids pitfalls and identifies the gaps in knowledge that are of greatest urgency [53]. A shift in approach for *S. neslonii* conservation such that published scientists are included in the funded projects will ensure that an increase in knowledge will begin to accompany every project.

5.2. Stop the Mortality

The urgent unanswered questions for *S. nelsonii* species recovery focus on plant mortality. First, the extremely high mortality of in situ seedlings on Guam and presumably on Rota leads to an unusual situation of considerable regeneration combined with recruitment failure [21,22]. One preliminary study has revealed the utility of horticultural experimental approaches to enable new knowledge about potential causes of seedling mortality within the Janzen–Connell hypothesis [40], and this research agenda must be expanded to understand this acute limitation to species recovery. Second, 60% mortality of the 1994 mature tree population has occurred during the 26-year implementation of the national recovery plan [42]. This has occurred with no direct observations by academic scientists, so the reasons for this attrition remain obscure. Species recovery will not be possible until this attrition phenomenon is studied with adaptive management research to illuminate mitigation protocols to stop the genetic erosion. Third, the widespread mortality of plants within historical in situ transplantation projects illuminates the need for adaptive management studies to improve post-transplant survival and growth. For example, the largest out-planting of nursery plants in the 1990s has led to 100% mortality with no adaptive management observational studies to illuminate the causes of mortality. According to regulations associated with the ESA [11], harming an individual of a listed species is prohibited. This literature review indicates that a nursery manager who fails to use repetitive stem pruning or some other treatment that increases the root:shoot quotient of the shade-grown transplants is proactively damaging the quality of *Serianthes* nursery plants by increasing post-transplant mortality. However, much more needs to be learned about post-transplant mortality, and the horticulture/silviculture literature contains numerous experimental approaches for continued research to reduce the mortality of the plants after removal from the conservation nursery.

5.3. Clone the Global Population

Every one of the initial 122 *S. nelsonii* trees should have been cloned in 1994 and conserved in replicated sites to ensure the preservation of the genetic diversity. As this did not occur, the remaining 49 mature trees should be cloned by competent horticulturists capable of using evidence-informed methods [19] to stop the ongoing genetic erosion. Much has been learned in recent years about how to capture in situ genetic diversity through the establishment of ex situ germplasm collections, and the use of clonal propagules captures more genetic diversity than seed propagules [54]. The cloned *S. nelsonii* individuals should be planted as subpopulation groups in replicated sites to conserve the genetic depth of the tree population as subpopulation units. Each of these managed subpopulation units could be available to serve as seed orchards [55] for the managed production of seeds for species recovery.

The use of grafting techniques also enables the production of single trees with multiple genotypes [56]. This approach could be used in *S. nelsonii* seed orchards to reduce self-pollination within single managed trees that are exploited for seed production.

5.4. Allow International Experts to Assess Species Recovery

The most recent multi-year recovery assessments [43,57] relied heavily on anecdotes and personal communications to promote a more-of-the-same approach for future recommendations. Relying on practitioner anecdotes and the gray literature can elicit conservation failures [12]. The lack of progress over the past 26 years does not justify a continuation of these same approaches for the future. Panels of international experts are often used to perform species recovery assessments to identify entrenched problems. Similarly, assessments of individual species extinction threats by the IUCN are ensured authenticity because they are conducted by international species experts. The use of these international approaches to assess *S. nelsonii* recovery efforts is long overdue, as reliance on the same military custodians, federal decision-makers, and practitioners will likely cause continued lack of progress toward species recovery.

5.5. Nurturing the Transition from Juvenile to Adult

The addition of 2000 reproductive trees within in situ locations will be required to meet the specifications of the national recovery plan for down-listing from endangered to threatened [4]. When seedling and sapling mortality limitations are removed through appropriate research by accomplished scientists, a subsequent research need will be to develop methods for nurturing an early transition from juvenility to maturity. Breeders and seed-producers in horticulture and silviculture have developed treatments that hasten the flower production of woody perennial angiosperm trees or the cone production of conifers. This literature is available to inform *S. nelsonii* conservation decision-makers, as the use of these techniques may allow earlier seed production on trees that are propagated for species recovery. Some of these treatments include bending of the apical stem region, the application of gibberellic acid, and girdling of the apical stem region [58–60]. This research could begin immediately by using these horticultural protocols to manipulate flower production on existing mature trees so that the most effective methods would be available for use on the managed juvenile trees when that stage of species recovery is achieved.

5.6. Graft All Dislodged Stems Following Tropical Cyclones

The death of *S. nelsonii* trees during tropical cyclones has been reported [14]. When trees are toppled or large limbs are broken, the dislodged stem material could be used to provide numerous scions for grafting many clones of the damaged tree [19]. This innovative use of the plant material could be enabled by the planting of hundreds of *S. kanehirae* seedlings in container nurseries at the end of each tropical cyclone season. These seedlings would be large enough for use as rootstocks throughout each subsequent tropical cyclone season. In the event that no stem damage to any of the existing *S. nelsonii* trees occurred during a tropical cyclone season, the *S. kanehirae* seedlings could be discarded or planted as street trees. This approach would allow the dislodged stems or toppled trees to contribute one last time to species recovery, rather than relegate the addition of their tissues to the detritus pool.

5.7. Give Different Teams a Chance

Why have decades of nursery production and out-planting projects failed to advance the goals of population expansion for species recovery? We believe this has occurred because the same practitioner team has been funded throughout the recovery plan's implementation. We advocate for a 12-month endeavor whereby various horticulture teams are allowed to use the new knowledge conveyed in this review to demonstrate their ability to produce high quality *S. nelsonii* transplants. The height, orthotropic orientation, and stem strength of the *S. nelsonii* plants could be objective metrics to quantify transplant quality from each team. The addition of *S. kanehirae* plants to the endeavor could allow destructive analysis to quantify the abilities of each horticulture team to nurture an elevated root:shoot quotient in the *Serianthes* nursery plants. This entire endeavor would reach culmination in 12 months, and the decision-makers would know which teams demonstrated the greatest level of skill for meeting the propagation mandate of species recovery. Considering the decades of failures of out-planting stock from *S. nelsonii* projects managed by the same practitioner team, a 12-month delay for identifying a team that is best equipped to supply high quality *S. nelsonii* stock is justifiable.

5.8. Ensure Healthy Restoration Sites Are Available

The vast literature on restoration ecology is useful for informing the methods for installing and sustaining new plantings of ESA-listed plant species. The decades-long history of the unsuccessful installation of *S. nelsonii* transplants is a conservation failure that is not unique to Guam. Indeed, many endangered plant and animal translocation attempts have failed for various reasons, and translocation ecology has emerged as a subdiscipline to study these successes and failures in threatened species conservation

science [61]. For example, priority effects whereby existing plant species influence the success of newly arriving species to a site profoundly determine the performance of the newly arriving species [62]. For this reason, recipient sites for the transplanted individuals of a threatened plant species should be as free of the negative influences of non-native species as possible in order to avoid legacy effects of the past disturbances [63]. A cost-effective strategy in ecological restoration may be the removal of non-native disturbing forces that have damaged a restoration site, then the provision of enough time for passive restoration to occur in the absence of the non-native disturbances [64,65]. For this reason, recommendations against the use of threatened plants within Guam's ecological restoration sites that have been degraded by non-native plant species have been communicated [66] because the legacy effects of those non-native disturbances may remain for many years. The current level of knowledge concerning plant-plant interactions [67,68] and plant-soil interactions [68,69] provides ample evidence that novel interactions of a native plant with a site disturbed by a history of non-native plants may not support the best health and longevity of the transplanted native plants.

6. Conclusions

When the *S. nelsonii* recovery plan was published, there were 122 trees in need of conservation, and the plan included the establishment of 2000 additional mature trees on two islands within a 16-year timeline. Successful implementation would have engendered more than 2000 trees today and the species would be down-listed from endangered to threatened status. We believe this goal was achievable if academic scientists with a strong publication record had been allowed to conduct appropriate adaptive management research from the start, and the new knowledge was used to guide the developing decisions.

The greatest need to improve *S. nelsonii* conservation is research to determine the reasons for 100% mortality of in situ seedlings, the widespread mortality of historical transplantation projects, and 60% mortality of the mature tree population during the 26-year implementation of the national recovery plan. Horticultural researchers are ideally positioned to answer these urgent questions. We believe that the continuation of the historical conservation approach to date will continue to propel this tree species toward an extinction vortex from which recovery will not be achievable.

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Review

Aulacaspis yasumatsui Delivers a Blow to International Cycad Horticulture

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Abstract: The literature covering the biology, invasion chronology, host plant responses, and control efforts of the armored scale *Aulacaspis yasumatsui* Takagi (Hempitera: Diaspididae) is reviewed. The small size of this cycad pest and complex surface morphology of the host cycad organs combine to make visual detection of every cryptic infestation difficult or impossible to achieve. The international movement of *Cycas revoluta* Thunb. nursery plants and the presence of *C. revoluta* nursery industries in so many countries have enabled this pest to wreak havoc on the international cycad horticulture trade over the last 25 years. The short pre-oviposition period and considerable female fecundity lead to rapid population expansion on the plants initially infested in newly invaded regions. A depletion of non-structural carbohydrates accompanies long-term infestations and precedes plant death. Enemy escape within the invasive range allows the scale population growth to remain unchecked until anthropogenic efforts establish non-native biological control.

Keywords: biological control; cycad; *Cycas micronesica*; invasion biology

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1. Introduction

Cycad aulacaspis scale, *Aulacaspis yasumatsui* (Hemiptera: Diaspididae), was first discovered in Thailand on *Cycas revoluta* plants in 1972, and was described in 1977 [1]. The scale was subsequently observed in other Southeast Asian countries and recognized as a *Cycas* L. pest. The accepted native range of *A. yasumatsui* is from the Andaman Islands to Vietnam [2], which includes Thailand, where the original collections were made that were used for the species description [1]. Within its native range, the infestations are not lethal to the host plants. The accidental introduction of *A. yasumatsui* to Miami, Florida in 1994 [2] led to widespread infestations; by the time the invasion had become common knowledge in 1996, the pest was recognized as a lethal threat to *Cycas* plants [3]. Typical horticultural protocols to control the scale were ineffective in Florida, and the insect rapidly extended its invasive range further [4].

Infestations of the scale in the presence of its natural enemies do not pose a serious threat to many cycad species, but in the absence of natural enemies, the infestations are lethal for most *Cycas* species. The international cycad horticulture nursery and landscape industries have been threatened by the pest's rapid invasion into many geographic regions. This continued expansion ultimately led to invasions into the native habitats of *Cycas micronesica* K.D. Hill [5] in Guam, and *C. taitungensis* Shen, Hill, Tsou and Chen [6] in Taiwan, immediately threatening the survival of both species. Within 10 years of the Florida invasion, the International Union for Conservation of Nature Cycad Specialist Group considered *A. yasumatsui* the single most important threat to natural cycad populations [2]. This single scale species demonstrates the immense cost and ecological impact associated with invasive species, which are increasing three-fold every decade [7].

These invasion events catalyzed the launch of research designed to understand the life history traits of *A. yasumatsui* and the responses of the host plants. Considerable effort has been invested in documenting the timing of the sequential invasions and various attempts at introducing biological control organisms. The objective of this review is to report on the *A. yasumatsui* literature produced over the last four decades since the organism was described. A review of the invasive nature of the pest since the Florida invasion may contribute to reducing the ongoing devastation of the cycad horticulture industry.

2. Search Methods

We conducted a Google Scholar search with "Aulacaspis yasumatsui" as the only query term. Based on a review of the search results, we sorted the articles into four broad categories, which defined the sections hereinafter. First, papers on the biology of *A. yasumatsui* were used to describe the traits unique to this insect (7 papers). Second, papers documenting the sequence of invasion in each country were tabulated in chronological order (30 papers). Third, papers on the plant responses by host plant individuals and populations were covered (39 papers). Finally, papers documenting the chemical or biological control programs in various countries were described (29 papers).

3. Biology

3.1. Life History

The first controlled trials conducted to understand the behavior of *A. yasumatsui* were carried out in Florida using clean *C. revoluta* plants exposed to the crawlers [3]. Under field conditions, scale densities of 70 per leaflet were observed after only 16 d of exposure, and females began egg production within 35 d. Total egg production was not assessed, but some female scale covers contained up to 110 eggs. Some eggs were incubated at 25 °C to determine the number of crawlers hatched in 8–12 d. This initial observational study revealed the high reproductive potential and speed with which the pest could threaten a host plant.

The first controlled study of *A. yasumatsui* ontogeny was conducted in Florida using *C. revoluta* as the host [8]. Use of a range of incubation temperatures revealed that an optimum temperature range of 30–32 °C resulted in an egg development time of 6.9–7.5 d. For females, nymphal development time was 19.7–21.6 d and total development time from egg to adult was 26.6–29.0 d. For males, the optimum temperature was 32 °C, at which nymphal development time was 18.0 d and total development time from egg to adult was 25.4 d. Extreme temperatures of 18 and 35 °C greatly inhibited development in the study. This experimental approach revealed that the scale's temperature tolerance range was broad, but some geographic locations with natural or cultivated populations may experience temperatures that suppress the development of *A. yasumatsui*.

The second study was conducted in Taiwan using *C. taitungensis* as the host [9]. At a constant temperature of 24 °C, egg incubation time was 7.3 d, female nymphal development time was 28.7 d, and total development time from egg to adult was 35.9 d. For males, total development time from egg to adult was 19.0 d. Maximum longevity of adult females during the oviposition period was 67.0 d, and for adult males was only 1 d. The net reproduction rate was 112 offspring per adult female.

One more study in Taiwan used *C. revoluta* as the host and employed a range of temperatures [10]. The optimum temperature was found to be 28 °C, resulting in an egg incubation time of 7.8 d. The durations of other stages were similar to previous reports; for females, the total development time from egg to adult was 36.8–59 d. and maximum longevity was 44.7–57.4 d. For males, duration of the adult stage was 1.5–2.1 d. The net reproduction rate per adult female ranged from 46 offspring at sub-optimal temperatures to 96 at optimal temperatures.

Field observations in Shenzhen, China found that up to eight generations of *A. yasumatsui* occurred during the warmer months of the year [11]. An observational study in Florida revealed

that freezing temperatures of −6.7 °C for 4 h were not lethal to *A. yasumatsui* on *C. revoluta* plants, and the scale infestations increased in density after the daily temperature rose [12].

3.2. Knowledge of Temperature Responses May Aid Cycad Conservation

Temperature-response studies [8,10] revealed that sub-optimal and supra-optimal temperatures were highly effective in reducing reproductive performance in *A. yasumatsui*. However, conservationists cannot rely on temperature extremes to eradicate this cycad pest, because it survives them even if it does not reproduce well at these temperatures. Still, planting ex situ germplasm collections in climatic zones with seasonal temperatures that are sub-optimal for the scale has been proposed as a passive approach for conserving endangered species such as *C. micronesica* and *C. taitungensis*, because adequate control of *A. yasumatsui* may be achieved with less control effort [10].

3.3. Organismal Research Needs

The combined results described in Sections 3.1 and 3.2 illuminate the ontogeny and fecundity traits of *A. yasumatsui* that cause such rapid lethal damage to the host plants. However, we believe there are several issues that require more research.

The two host plants used in the published studies were *C. revoluta* and *C. taitungensis*. These species are among the most susceptible in the context of rapidity of death after the initial scale infestation. In most botanic gardens and nurseries, the scale can be found feeding on other cycad taxa that are not at risk of lethal damage (see Section 5.1). Nothing is known to date about the performance of *A. yasumatsui* when feeding on one of the less palatable cycad taxa. Horticulturists and conservationists urgently need this information to define the most appropriate mitigation protocols in a common garden setting.

The phenotypic heterogeneity of *A. yasumatsui* appears to be greater than that of other *Aulacaspis* taxa [2,13]. This may be due to genetic diversity in *A. yasumatsui*, as has been shown in other insects. For example, study of the genetic structure of the cycad specialist butterfly *Luthrodes pandava* Horsfield has identified four distinct cryptic subspecies [14]. More studies are needed to determine whether this heterogeneity in *A. yasumatsui* is due to the existence of cryptic species, or differences in environmental factors or in the nutritional value between various host plants. Moreover, the scale's endosymbiont diversity is influenced by diet [15], and more research is needed to determine whether *A. yasumatsui* endosymbiont diversity may influence its phenotype.

Our understanding of the *A. yasumatsui* crawler stage is inadequate. This stage is of crucial importance for several reasons. *Cycas* plants infested with *A. yasumatsui* may develop high-density infestations as a result of crawler behavior. A peculiar result of this behavior is that plants that are initially infested in a newly invaded habitat may become completely covered by *A. yasumatui* before the adjacent plants begin to be infested [16]. The crawler is the only developmental stage in armored scales that can achieve medium- to long-distance passive locomotion by exploiting wind currents or by hitch-hiking on passing animals including humans, a process known as phoresis [17]. For example, after *A. yasumatsui* began rapidly expanding its range on Guam in 2005, new infestations developed that were disjunct such that wind or proximity to infestated sites could not explain the pattern of population expansion [18]. Similarly, the island of Tinian has many *C. micronesica* and *C. revoluta* plants in the managed urban landscape and a large, publicly funded ex situ *C. micronesica* germplasm collection in a remote forest within a federally restricted area. The island was invaded by *A. yasumatsui* in August 2019 [19] and the initial infestation occurred in the remote protected area, on the access trail entering the expansive cycad collection. This Tinian invasion had been predicted [20] because the recent conservation decisions included sending a maintenance team from Guam, where *A. yasumatsui* infestations are ubiquitous, to Tinian every month. The only biological explanation for this island invasion and its initial location was that biologists with permission to enter the remote protected area had vectored phoretic crawlers into the site.

Aulacaspis yasumatsui crawlers become sessile within 1.0–1.7 d if suitable feeding sites are readily available [8,10]. However, nothing is known about how many days a crawler can remain viable if a suitable feeding site is not immediately available. This is undoubtedly less than the duration of the first nymphal instar, which may be as short as 9 d at optimal temperatures and as long as 31 d at sub-optimal temperatures [8,10]. Replicated trials are urgently needed to withhold host tissue from crawlers for various durations of time before providing them with host tissue in order to determine whether they can become established and reach maturity. Such trials would determine how long a crawler carried by wind or through phoresy may remain potentially infective.

4. The Expanding Invasion Range

The movement of live cycad plants containing undetected infestations of *A. yasumatsui* is the means by which invasions have likely occurred in new geographic areas. The most popular cycad species for horticultural use is *C. revoluta*, and this versatile plant is ideal for many horticultural applications (Figure 1). The main reason that infestations may be undetected is because the small, sessile insects settle in crevices and convoluted surfaces that make direct observance of some individuals difficult or impossible [21]. A single, hidden gravid female could generate more than 100 offspring in several weeks after a *Cycas* plant is passed through quarantine. Unfortunately, *C. revoluta* is one of the species that provides *A. yasumatsui* with numerous cryptic feeding sites not observable during direct inspection of the intact plant (Figure 2a). Moreover, cycad roots can serve as feeding sites for *A. yasumatsui*, and infestations may occur down to 60 cm below the soil surface [4]. At the time of the initial Florida invasion, much of the *C. revoluta* horticulture industry was comprised of container-grown nursery plants [2], for which detection of root infestations within the container medium was not possible.

(**a**) (**b**)

Figure 1. *Cycas revoluta* is shipped in high volumes to support the international horticulture industry. The species is used in numerous horticultural applications. (**a**) Containerized specimen plants are used as house plants, accent plants in the landscape, and bonsai. (**b**) Mass plantings of *C. revoluta* provide stunning landscapes.

(**a**) (**b**) (**c**)

Figure 2. *Aulacaspis yasumatsui* can infest many surfaces on *Cycas* host plants that are impossible to see during visual inspections. (**a**) *Cycas revoluta* stem section reveals the overlapping persistent leaf bases that create cavities where the armored scale may persist. (**b**) Apex of *C. tansachana* K.D. Hill and S.L. Yang stem covered by dense trichomes that can conceal scale infestations. (**c**) Mature megastrobilus of *C. nongnoochiae* K.D. Hill revealing numerous cavities in which scale infestations may be hidden.

Ambiguities have plagued the documentation of some country invasions, primarily because the live appearance of *A. yasumatsui* is similar to many other armored scale species [22]; this can lead to difficulty in diagnosis. Authoritative identification requires microscopic study of slide-mounted adult females. The case of Indonesia provides an example of the need for identification by a taxonomic authority; the probable invasion prior to the 1980s [23,24] remains equivocal because *A. yasumatsui* was not authoritatively identified in the country by a taxonomic expert until a 2011 outbreak [25]. This phenotypic similarity to other scale species may lead to identification mistakes by quarantine officers, so molecular diagnostic techniques have been developed to aid in species identifications [26].

The initial confirmed identification of *A. yasumatsui* on cycad plants outside the scale's native range occurred in 1994, when infested plants were observed in Miami, Florida [2] and 1995, when plants imported from Vietnam to the Netherlands were infested with the scale [6]. If the *A. yasumatsui* infestation on imported plants which caused the Florida outbreak had been detected at the port of entry, there is a chance that the Florida invasion could have been prevented. At the time of the invasion, the *C. revoluta* nursery industry in Florida was substantial, with container-grown plants being shipped to numerous other states and countries in high volumes [2]. The invasive range of the pest expanded dramatically within a few years as a result of this trade in whole plants. We list the many invasions and interceptions that have been reported by the year of first report (Table 1).

Table 1. List of geographic locations which have reported *Aulacaspis yasumatsui* on established cycad plants or on plants intercepted at a port of entry. The list does not include countries within the unambiguous native range of the scale.

Year	Location	Citation
1992	China	[27]
1994	Continental United States	[2]
1995	Netherlands	[6]
1998	Hawaii	[27]
1996	Cayman Islands	[2,28]
1996	Hong Kong	[2,27]
1996	U.S. Virgin Islands	[2,28]
1996	Puerto Rico	[2,29]
1996	Saint Kitts	[2]
1996	Singapore	[2,27]
1996	Taiwan	[2,6]
2001	France	[30,31]
2001	Singapore	[27]
2003	Barbados	[6]
2003	Guam	[32]
2003	Guadeloupe	[33]
2004	Costa Rica	[6]
2004	New Zealand	[6]
2006	Croatia	[34,35]
2006	Ivory Coast	[6]
2006	United Kingdom	[6]
2007	Rota	[18]
2008	Palau	[36]
2008	Philippines	[37]
2009	Bali	[24]
2009	Bulgaria	[38,39]
2009	Java	[24]
2009	Poland	[40]
2009	Timor	[24]
2012	Malaysia	[25]
2012	Sulawesi	[41,42]
2013	Cyprus	[43]
2014	Nigeria	[44]
2015	Mexico	[45]
2015	South Africa	[46]
2015	Turkey	[47]
2016	Guatemala	[48]
2018	Dominican Republic	[49]
2019	Tinian	[19]

This list of reported country invasions is assuredly incomplete because some invasions may have been reported in literature that was not identified in our systematic search; many infestations or quarantine confiscations may remain undetected or unreported; or the *A. yasumatsui* infestations were misidentified as another scale species. Moreover, in many cases the date of first publication is known but the initial date of invasion is impossible to determine because this was not reported. However, the list exemplifies several critical issues of importance to horticulture and invasion biology. First, when the recent pattern of expanding invasive range is studied, locations that are vulnerable to impending invasion risk may be apparent. The 2003 invasion of Guam [50] and 2019 invasion of Tinian [20] were both predicted before they were reported. Second, newly invaded geographic locations may serve as conduits through which continuing invasions occur; for example, exports from the continental United States and Costa Rica were the likely sources of invasions into Europe and Africa. Third, residents of isolated islands can be successful in keeping out invasive species when decision makers are informed and sound science is respected.

For example, the residents of the Mariana Island of Tinian were successful in keeping this lethal cycad pest from invading their island for decades after the international *A. yasumatsui* expansion began, and the invasion did not occur until 2019 as a result of an ex situ conservation project [19].

The considerable data available have been used to develop models of future invasion potential for *A. yasumatsui* [51,52]. These models and the 25-year history of documented invasions may be used to improve policy and action plans for managing the continuing expansion of the invasive range of this devastating horticultural pest.

5. Host Responses

5.1. Host Range

All cycad species within the native range of *A. yasumatsui* belong to the genus *Cycas*. The 25 years of interactions of this scale with many cycad species in the invasive range indicate *Cycas* species are the best quality hosts for *A. yasumatsui* growth and fecundity. *Cycas* species are also among the most susceptible to damage by the scale. The range in susceptibility among cycad taxa is relevant to the horticulture industry because maintaining a suitable-looking specimen is often difficult despite copious amounts of intervention. Without biological or chemical control, the susceptible cycad species die rapidly after the scale population reaches high densities. Many owners of cycad plants in non-commercial locations, after several years of unsuccessfully trying to maintain the beauty of the cycad specimen, give up and resort to removing the cycad plants. For these reasons, the full list of host species of *A. yasumatsui* should be understood by horticulturists and conservationists.

The current understanding of cycad diversity recognizes 364 distinct species, 120 of which belong to the monogeneric Cycadaceae family [53]. Every Cycadaceae species that has been observed is a suitable host for *A. yasumatsui*. We list the Zamiaceae species which are known hosts, based on direct observations (Table 2).

Table 2. List of Zamiaceae species that are reported as hosts for *Aulacaspis yasumatsui*. The list is developed from the literature and from the authors' personal observations.

Species	Taxonomic Authority
Bowenia serrulata	(W. Bull) Chamb.
Ceratozamia robusta	Miq.
Dioon califanoi	De Luca and Sabatori
Dioon edule	Lindl.
Dioon merolae	De Luca
Dioon mejiae	Standl. and L.O. Williams
Dioon rzedowskii	De Luca, Moreti, Sabatori and Vasquez
Dioon sonorense	(De Luca et al.) Chemnick et al.
Dioon spinulosum	Dyer ex Eichler
Encephalartos barteri	Miguel
Encephalartos ferox	G. Bertol
Encephalartos hildebrandtii	A. Bran and C.D. Bouché
Encephalartos manikensis	Gilliland
Encephalartos pterogonus	R.A. Dyer and I. Verd
Encephalartos whitelockii	P.J.H. Hurter
Macrozamia lucida	L.A.S. Johnson
Macrozamia miquelii	F. Muell.
Microcycas calocoma	(Miq.) A. DC.
Stangeria eriopus	(Kunze) Baill.
Zamia loddigesii	Miq.
Zamia integrifolia	L. f.

5.2. Individual Plant Responses

The reserves of non-structural carbohydrates in *C. revoluta* leaves, stems, and roots decline increasingly in proportion to the length of exposure to *A. yasumatsui* herbivory [54]. Starch exhibits greater relative declines than sugars, and disaccharides exhibit greater

relative declines than hexoses. The ongoing depletion of carbohydrates with duration of herbivory is probably how *A. yasumatsui* kills the host plant.

In Guam, highly disparate germination performance of *C. micronesica* seeds among numerous habitats was observed, whereby the percentage germination of seeds that were free of *A. yasumatsui* infestation was six times greater than that of seeds directly infested by the scales [55]. These observations led to manipulative studies which determined that the carbohydrates in gametophyte and sarcotesta tissues were greatly affected by *A. yasumatsui* herbivory. The greatest relative decline in carbohydrates during *A. yasumatsui* seed infestation occurred in the sarcotesta tissue. Scale-infested seeds contained a gametophyte starch pool that was only 37% of that of uninfested seeds. The ability of *A. yasumatsui* to deplete the non-structural carbohydrates of host plants clearly affects consequential transgenerational phenomena.

Two cascading effects of reduced carbohydrate status due to long-term *A. yasumatsui* herbivory have been studied. First, the use of stem cuttings from scale-damaged trees for producing new *C. micronesica* plants is less successful. For example, the success rates for adventitious root formation on cycad stem cuttings is in excess of 90% for experienced cycad horticulturists if the source plants are healthy [56–59]; however, success rates declined to only 30–40% after the source trees had been damaged by *A. yasumatsui* for seven years [56,60]. Second, stem CO_2 efflux of scale-damaged *C. micronesica* plants was reduced when compared to that of healthy trees [61]. This simple non-destructive field measurement was proposed as an indirect approach for estimating the stem carbohydrate status of live but unhealthy trees, which may improve selection of the best candidates for obtaining stem cuttings for asexual propagation.

Height increment of cycad plants has not been adequately studied. The plants are relatively slow to increase in height when compared to most arborescent species. The height increment of uninfested *C. micronesica* was 2.5–3.0 $cm \cdot y^{-1}$; this was reduced by chronic *A. yasumatsui* damage to 1.6 $cm \cdot y^{-1}$ [62].

Branching behaviors of cycad trees are unique, and many female trees remain unbranched for life. In contrast, most male trees exhibit several points of dichotomous branching [63]. The pre-invasion Guam populations of *C. micronesica* were comprised mostly of unbranched trees, but after 15 years of *A. yasumatsui* damage most of the remaining live trees contained three or more branches. The long-term mortality of this insular cycad species has revealed female trees were killed by *A. yasumatsui* more often than male trees.

5.3. Population and Habitat Responses

The most researched case study after an *A. yasumatsui* invasion has been that of *C. micronesica*. Three Guam studies used permanent plots to obtain population data over time. First, belt transects were established in a site in northwest Guam before *A. yasumatsui* invaded the site in early 2005. This was a high-density *C. micronesica* forest where many biochemistry and pollination studies were conducted; it contained the most genetically isolated *C. micronesica* stand on Guam [64]. Survival and regeneration data were obtained until 2011 to develop a Type I right-censoring approach [65]. This six-year study revealed for the first time that seedlings and small juvenile plants were killed by *A. yasumatsui* herbivory more rapidly than large plants. Seedlings were killed within nine months, and juvenile plants less than 100 cm tall were killed within 40 months. Some regeneration occurred in the earliest years, but every seedling was killed so by 2008, no recruitment was occurring [66]. Mortality of 92% occurred within the six years of *A. yasumatsui* infestation. The study site was abandoned in 2015 when *C. micronesica* was added to the United States Endangered Species Act [67], as the federal permitting and access regulations became inhibitory.

Second, numerous plots were positioned throughout Guam in 2005 prior to the arrival of *A. yasumatsui* in each area. All the research sites on federal property were abandoned in 2015 due to new federal restrictions on access, but the remaining 12 sites

were monitored annually until 2020 to provide 15 years of survival data [68]. These long-term data corroborated the results of the first study, in that seedling mortality was 100% by 2006, juvenile plant mortality was 100% by 2014, and the census after 15 years of *A. yasumatsui* damage revealed 96% mortality of the *C. micronesica* population.

The third study was a forest inventory conducted in 2002 [69] and repeated in 2013 [70]. The 2002 inventory revealed *C. micronesica* was the most abundant tree species on the island, with an estimated population of 1.57 million trees [69]. By 2013, the population had declined to 0.62 million trees, leaving 20 other arborescent taxa more abundant than *C. micronesica* [70]. Interestingly, *Cocos nucifera* L. was the second most abundant tree species on Guam in 2002, and this native species also declined in population such that by 2013, it ranked 15. This decline in the coconut tree population was a result of the 2007 *Oryctes rhinoceros* L. invasion [20]. Therefore, the two most abundant tree species on Guam in 2002 were decimated by non-native specialist insect invasions in the period 2003–2007 such that by 2013, neither ranked in the top ten. An estimated 93% of the *C. micronesica* trees exhibited *A. yasumatsui* infestations in 2013 [70].

The influence of *A. yasumatsui* on a host plant cannot be fully understood without attempting to understand the plant's interactions with other native or non-native herbivores [71]. For *Cycas* species, the most common co-occurring herbivores are the specialist butterfly *Luthrodes pandava* (synonym: *Chilades pandava* Horsfield) [71,72] and the mutualistic pollinator species [73–75]. The most researched *A. yasumatsui* invasion example is the Guam *C. micronesica* case study. The 2003 Guam invasion by *A. yasumatsui* was accompanied by other invasions, of *L. pandava* (which feeds on young, expanding leaf tissue), *Erechthias* sp. Meyrick moth (which mines old *Cycas* leaflets), and *O. rhinoceros* (which bores into the stems) [5,20,76]. Previously non-threatening damage to *C. micronesica* by the native stem borer *Acolepta marianarum* Aurivillius and feral pigs (*Sus scrofa* L.) added to the threats to the trees because their health was compromised by the invasive insects [20]. The direct interactions of *A. yasumatsui* and the native *Anatrachyntis* Meyrick pollinator [77,78] have not been studied; however, chronic damage by *A. yasumatsui* and the other non-native pests decimated the tree population [68], and this indirectly damaged the native pollinator because *Cycas* male cones provide brood sites for pollinator regeneration [5,77,78]. Historically, hundreds of *Anatrachyntis* adults emerged from each *C. micronesica* male cone after pollen dispersal, but recent attempts to rear pollinators from *C. micronesica* male cones on Rota Island have yielded no pollinators (unpublished, T.E.M.). These observations indicate that the pollinator population may have been extirpated on this island due to the infrequent production of male cones because of damage by invasive insects. The Guam case study provides examples of invasional meltdowns from the long list of invasive herbivores and coextinction threats due to loss of pollinator species [79].

Direct interactions of several insects have been studied in the Guam case study [20,80]. For example, *Erechthias* damage declined but *Acolepta marianarum* damage increased after *Aulacaspis yasumatsui* damage. Negative correlations between *A. yasumatsui* and *L. pandava* indicate that these two insects are in direct competition when *C. micronesica* is their only host.

Cycas micronesica associates with nitrogen-fixing endosymbionts and improves ecosystem health by introducing nitrogen into the soil food web and increasing heterogeneity in biogeochemistry [81]. One of the unique traits of the tree is slow leaf-litter decomposition compared to other sympatric tree species [82]. However, the litter quality of *C. micronesica* leaves was altered by *A. yasumatsui* herbivory such that nitrogen and potassium concentrations increased, changes that predicted an increase in the decomposition speed of the *C. micronesica* leaf litter [83].

The soils adjacent to *C. micronesica* trees damaged or killed by *A. yasumatsui* are plagued by a phytotoxic legacy that damages understory plant growth [84]. The soils may remain barren for years, and experimental manipulations indicate the presence of persistent organic substances may be causal. This legacy effect may be related to large numbers of persistent dead *A. yasumatsui* bodies and scale covers on the *C. micronesica* leaf litter [85].

Multiple stressors imposed on plants simultaneously may produce unique challenges for plants in a manner that cannot be predicted by studying individual stressor responses [86]. Interactions between pests and other forest disturbances are instrumental in driving many forest dynamics [87]. The Guam case study has contributed to the examples of these ecological phenomena, whereby the intrinsic resilience of *C. micronesica* trees to the region's frequent tropical cyclones [88] is compromised by chronic *A. yasumatsui* herbivory [89–91].

Consequential interactions between *A. yasumatsui*, the predator *Rhyzobius lophanthae* Blaisdell, and the host *Cycas* plants may influence the efficacy of the predator. The cryptic location of some *A. yasumatsui* infestations [21] may protect some *A. yasumatsui* individuals from access by the predator [92]. For example, the overlapping persistent leaf bases, dense trichomes, or long-lived reproductive structures on *Cycas* plants (Figure 2) provide small crevices where the armored scales can live but the predator cannot enter. This phenomenon increases the value of parasitoid biological control because the size differential between the scale and the ovipositing parasitoid is minimal. In addition, for unknown reasons the predator preferentially forages at higher levels, and prefers to avoid the strata near the soil surface [16,93]. This predator behavior may result in localized *A. yasumatsui* infestations on leaves near the soil surface on plants that exhibit no *A. yasumatsui* on leaves in the higher strata. Finally, when provided a binary choice in an olfactometer assay, this predator prefers *A. yasumatsui* on mature tree leaves versus seedling leaves [94].

6. Control Methods

6.1. Chemical Control

Numerous trials have been conducted to determine the efficacy of various insecticides for *A. yasumatsui* control; most have been ineffective in defining an economical approach. However, the crawler stage of this pest appears to be vulnerable to most contact insecticides including horticultural oils and soaps. Use of contact insecticides for controlling *A. yasumatsui* requires vigilance, with an application frequency of less than weekly, and attention to saturation of every plant surface with each scheduled spray in order to contact every crawler. The more efficacious active ingredients identified in the initial trials for use as frequent spray applications were pyriproxyfen [22] and dimethoate [95]. Soft approaches for suppressing *A. yasumatsui* infestations have been communicated also. For example, the use of coffee grounds for suppressing *A. yasumatsui* was promoted in Florida [96], but these assertions were not corroborated in a replicated trial [97]. Howard et al. [3] were the first to determine the efficacy of imidacloprid for controlling *A. yasumatsui*, and this systemic insecticide may require the least amount of labor for chemical control of the scale. This systemic insecticide has been used for *A. yasumatsui* control on *C. micronesica* to enable in situ research on horticultural and physiological questions [55,56,91,98].

6.2. Predator Control

The greatest concerns with the continuing international spread of *A. yasumatsui* involve conservation of in situ cycad populations, which must rely on biological control. Biologists in many geographic locations have indicated that pre-existing biological control organisms may fortuitously begin to attack the newly invaded *A. yasumatsui* populations. Other teams have purposefully introduced biological control organisms to control a new *A. yasumatsui* invasion. Organized efforts to introduce predators or parasitoids to locations that have been invaded by *A. yasumatsui* have been ongoing since 1997. The predators of the scale that have been reported are listed in Table 3.

Table 3. A list of predators known to feed on *Aulacaspis yasumatsui*.

Species	Family	Case Studies
Chilocorus cacti	Coccinellidae	[29,99]
Chilocorus circumdatus	Coccinellidae	[99]
Chilocorus stigma	Coccinellidae	[99]
Curinus coeruleus	Coccinellidae	[99]
Cryptolaemus montrouzieri	Coccinellidae	[29,99]
Cybocephalus sp.	Cybocephalidae	[100]
Cybocephalus nipponicus	Cybocephalidae	[25,33,99,101–111]
Cybocephalus flavocapitis	Cybocephalidae	[104,107,108]
Cycloneda sanguinea	Coccinellidae	[99]
Diomus austrinus	Coccinellidae	[99]
Exochomus children	Coccinellidae	[99]
Harmonia axyridis	Coccinellidae	[99]
Hyperaspis ornatella	Coccinellidae	[99]
Hippodamia convergens	Coccinellidae	[99]
Microweisea coccidivora	Coccinellidae	[99]
Olla v-nigrum	Coccinellidae	[99]
Phaenochilus kashaya	Coccinellidae	[109,110,112,113]
Rhyzobius lophanthae	Coccinellidae	[25,99,101,102,105,109,110,114,115]
Zilus subtropicus	Coccinellidae	[99]

6.3. Parasitoid Control

Entomologists are skilled at searching for predators and parasitoids within armored scale infestations, but most gardeners and horticulturists are not trained in detecting the signs that an armored scale has been parasitized. This may explain why the list of parasitoids that are known to parasitize *A. yasumatsui* is much shorter than the list of predators (Table 4).

Table 4. A list of parasitoids known to parasitize *Aulacaspis yasumatsui*.

Species	Family	Case Studies
Ablerus sp.	Aphelinidae	[29]
Aphytis lingnanensis	Aphelinidae	[20,105,109,110]
Coccobius fulvus	Aphelinidae	[8,12,28,29,32,33,101,105,109,110,116,117]
Encarsia sp.	Aphelinidae	[99,118]
Encarsia diaspidicola	Aphelinidae	[119]
Pteroptrix sp.	Aphelinidae	[110]
Arrhenophagus chionaspidis	Encrytidae	[8,20,25,109,110]
Aprostocetus sp.	Eulophidae	[118]
Aprostocetus purpureus	Eulophidae	[8,99]

6.4. Other Biological Enemies

In addition to these predators and parasitoids, the entomopathogenic fungus *Isaria fumosorosea* Wize infects *A. yasumatsui* and may be useful for controlling the scale [120]. Moreover, the nematodes *Steinernema feltiae* Filipjev, *Heterorhabditis indica* Poinar, Karunaka and David, *Heterorhabditis marelatus* Liu and Berry, and *Heterorhabditis bacteriophora* Poinar may be useful for suppression of *A. yasumatsui* [99,101].

7. Lessons Learned and Future Directions

Many geographic locations in tropical and subtropical regions have relied on *C. revoluta* as a ubiquitous part of the urban landscape. The species is famous for providing stunning specimen plants requiring minimal to no care. It is also famous for possessing a toolbox to thwart every threat; but within months of an *A. yasumatsui* invasion into one of these locations, *C. revoluta* and other *Cycas* species in the landscape lose their horticultural appeal. With a few exceptions (such as the original 1994 Florida invasion and the 2019

Tinian invasion, where publicly funded conservation projects were instrumental in the invasions), the international trade in infested horticultural plants was responsible for most of the other invasions.

Enacting a conservation action plan for an endemic *Cycas* population that becomes threatened by a new *A. yasumatsui* invasion requires an understanding of myriad interacting phenomena. Conservation mistakes can be made if decision-makers are not open to input from experienced experts. For example, most of the funds for conserving *C. micronesica* on Guam have been invested in expensive propagation and tree-rescue projects. However, the primary threat to the species is herbivory by non-native insect herbivores. This threat remains unchanged by the expenditure of the conservation funding [62,121] and if the public funds were instead directed toward control of the insect threats rather than tree-rescue projects, the chance of *C. micronesica* species recovery would improve.

Aulacaspis yasumatsui was thought to be the only member of *Aulacaspis* Cockerell associated with gymnosperm host plants. However, four other *Aulacaspis* species have been identified that use *Cycas* plants as hosts: *A. madiunensis* Zehntner, *A. mischocarpi* Cockerell and Robinson, *A. rosae* Bouché, and *A. zunyiensis* Wei and Jing [122–125]. *Aulacaspis madiunensis, A. mischocarpi* and *A. rosae* are oligophagous to polyphagous but the full host range for *A. zunyiensis* is not known. *Aulacaspis yasumatsui* appears to be host-specific to Cycadaceae and Zamiaceae taxa.

The heavily discussed native habitats of endemic cycad species that have been threatened by the international list of *A. yasumatsui* invasions were on the islands of Guam, Rota, and Taiwan. Lack of fortuitous biological control and the initial *C. micronesica* and *C. taitungensis* plant mortalities were well-documented following these invasions. Very little has been discussed about the interesting case of the Philippines, an archipelago that is outside the native range of *A. yasumatsui* but is home to 13 accepted *Cycas* species [53]. *Aulacaspis yasumatsui* is known to occur in the country [37] but the original invasion date is not known. Since the Philippines has unusually high *Cycas* diversity within a small geographic range, more research is needed to develop the necessary knowledge to conserve these unique cycad species [37].

Rapid identification of the armored scale in every newly invaded location should be conducted by an experienced taxonomist, and is mandatory for developing a rapid action plan. For example, the Miami invasion caught cycad conservationists and horticulturists by surprise because the initial anecdotal identifications indicated that the scale outbreak was the locally ubiquitous *Pseudaulacaspis cockerelli* Cooley [22]; this lessened the success of early attempts at control.

Rapid establishment of biological control is also required to thwart initial *A. yasumatsui* establishment, especially in insular settings. The Guam invasion was observed in 2003, but the first release of a predator was not permitted and enabled until 2005. The slow pace of the regulations for introducing biological control on Guam is an example of how delays in establishing biological control can contribute to ecosystem damage [126].

Initial reports suggested imidacloprid was effective in suppressing *A. yasumatsui* populations. However, long-term use appeared to generate resistance to this chemical. Observational knowledge that has accrued in Florida and Thailand cycad nurseries and private collections has revealed the use of pyrethroid insecticides such as bifenthrin and cypermethrin and/or organophosphate insecticides such as acephate and chlorpyrifos [127–129] are more efficacious (personal observation, A.L.). The development of resistance to any one insecticide appears to be rapid, and replicated trials are needed to determine an optimum interval for scheduling alternations among the efficacious insecticides.

Publicizing a new country invasion is required to enable the most effective international response [2]. An inadvertent or purposeful decision to withhold news of a new *A. yasumatsui* invasion from the international community is a decision to deny threatened cycad plants the international expertise and protection that they deserve. This recently occurred on the island of Tinian, where the news of an August 2019 invasion by *A. yasumatsui* was not made public until 19 months later [19]. The confusion about the date of

the first *A. yasumatsui* invasion of Indonesia [23–25] provides an example of how initial identification by a taxonomic authority and rapid communication to the international community are crucial steps to bring clarity to this international problem.

Most cycad species are remarkably resistant to threats, and resilient following abiotic or biotic damage in their native habitats. This is one of the endearing traits of the plant group that attracts plant collectors as they learn to cultivate cycad plants. Horticulturists that are new to cycads often kill their first few plants with too much attention, then become more knowledgeable about the need to leave the plants alone to appreciate their persistent traits [130]. However, when a *Cycas* population is invaded with *A. yasumatsui* the plants do not possess the tools to fight back, and the initial infestation often signals a one-way trip toward death. This acute host-herbivore bipartite system contains the answers to many questions of interest to biologists. However, Guam is only one of 39 reported invasions (Table 1), yet almost all of the studies to determine what this armored scale does to its host have been conducted on Guam (see Sections 5.2 and 5.3). These oversights should be corrected by biologists in other invaded regions in order to best understand how to conserve the host cycads from the threats imposed by this armored scale.

8. Conclusions

The invasion of Florida by *A. yasumatsui* 25 years ago initiated a sequence of invasions that have devastated horticulture industries based on *Cycas* species, and threatened the in situ populations of two *Cycas* species. Many lessons have been learned during this time, and the knowledge that has been generated is available to inform future conservation decisions. The small size of this cycad pest and the complex surface morphology of the host cycads make low-density infestations impossible to detect by visual inspection. The short pre-oviposition period and considerable female fecundity of *A. yasumatsui* lead to rapid population expansion on the plants that are initially infested in newly invaded regions. Host plants succumb to the herbivory through gradual depletion of non-structural resources. Enemy escape within the invasive range allows explosive scale population growth, requiring resident biologists to enact a rapid plan to search for fortuitous pre-existing natural enemies and to introduce predators or parasitoids from other invaded regions where biological control has been successful. International trade is responsible for many of the world's devastating invasions [131], and our case study provides a compelling example of how greater regulation of the international horticulture industry may aid in curbing the global risks of some invasive species.

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Review

Biological Contribution of Ornamental Plants for Improving Slope Stability along Urban and Suburban Areas

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Abstract: Plants can reduce erosion during heavy raining periods and improve slope stability through their root morphology, development, biomass, and architecture. Heavy rains can increase erosion, becoming a danger for traffic and people who live around slopes. The control of slope stability is often required in urban and peri-urban environments, and for this reason ornamental species can be appropriately selected for a dual use, namely improving the aesthetical value of green areas along the urban and suburban roads and mitigating the erosion effects. The species used must have good tolerance to abiotic stresses, such as high and low temperature, drought, pollution and nutrient deficiency. Otherwise, their limited growth can reduce their beneficial effects. Ornamental plants that can be used for reducing the erosion of slopes must be in full growth during periods with a higher incidence of rains and must also be compatible with the temperature ranges in different seasons. These species can be also selected for their ability to avoid erosion and enhance the stability of slopes. In this review, the biological contribution of plants for improving slope stability has been reported and discussed with a special focus attention on the Mediterranean environment. Particular emphasis has been placed on root biomass changes and root growth parameters, considering their role as potential markers for selecting suitable plants to be used for enhancing slope stability. A brief description of planting on slopes and root growth has been also considered and discussed.

Keywords: root development; root morphology; abiotic stress; growth regulators; biostimulants; plant choice

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1. Introduction

Urban and peri-urban green areas provide important ecosystem services for the quality of the urban environment such as air pollution mitigation, direct effects on local climate, noise abatement, stormwater management during rainy periods, carbon dioxide assimilation, oxygen supply, and recreational and social benefits [1]. Turfgrass, ornamental shrubs, and trees, can deliver different ecosystem services beyond their aesthetical contribution depending on the composition and biodiversity [2]. They are also used for improving the stability of slopes along roads and in urban areas [3]. The stability of slopes is mainly due to plants' root biomass, distribution, and architecture [4]. The current review has the objective of highlighting the biological contribution of ornamental plants to increasing slope stability. Through a review of the literature, the current work will explain how ornamental plants (with their habitus, growth, and roots systems) can prevent erosion and improve slope stability in urban and suburban areas.

Roots grow in soil and create an underground net able to reduce or avoid erosion and landslides that can be dangerous and induce severe damages in urban environments.

Plant density and biodiversity affect both the number and architecture of roots and their contribution to the slope stability through hydrological and mechanical processes [5]. Root morphology and growth are influenced by genetic background, soil characteristics, and climate conditions (i.e., prevailing winds) [5]. The root morphology, in terms of diameter or ramification, can enhance the soil held back and the shear-strength of the rooted soil; a higher root diameter has beneficial effects on soil stability, acting as strong underground grid. Moreover, roots can improve water retention during the raining period and create a drainage network that allows for soil water absorption, avoiding runoff and erosion [6]. The soil around the roots is hydrologically and mechanically more stable and the consequences of this are easier infiltration, better physical and chemical properties of the soil, and higher shear strength [7]. All of these factors can positively or negatively affect soil erosion [8]. Factors reducing the soil erosion are mainly represented by vegetation or physical barriers [9]. The effect of vegetation on erosion control can be dramatically observed in areas subjected to fire events, where the massive destruction of plants and their roots system cannot hold the soil in slope conditions with high incidence of landslides. Roots are living organs and are subjected to turnover. Therefore, dead roots generate empty channels useful for drainage [6,10].

Slope stability also depends on the roots' depth, uniformity, and distribution. Different plant species should be closely planted, and the selection of species should be conducted with regard to the root distribution in soil and their interactions, avoiding those that can have antagonist responses. Plants with deep root systems should be associated with species having superficial roots, providing a good root network at different depths [10]. The stability of soil in planted slopes depends on the interaction of roots of different species that can synergistically work or have antagonist effects. Some allelopathic compounds could reduce the efficacy of the plants to prevent erosion or landslides. Therefore, plant selection for slope greening must be accurately carried out. A wrong plant species combination can limit plant growth and benefits, and this can be also a disadvantage for slope stability [11].

However, plant leaf area, branch density and ramification can reduce the energy of precipitation (soil impact) of rain and runoffs. The combination of turfgrass, shrubs, and ornamental trees can simultaneously reduce the kinetic force of rain with multiple canopies at different heights and water run-off is slowed down by grass [12]. The reduced superficial run-off velocity increases soil water infiltration exploiting, the channels created by the roots of different species. In evergreens, the canopy can also reduce the snow accumulation on the slopes avoiding possible landslide events. Evergreen shrubs or trees can also remove the water from the soil through transpiration (even in winter) and by reducing landslides (even if low temperatures slow down plants' metabolism). Slopes subjected to landslides should be preferentially covered by evergreens, these being deciduous plants with inactive roots during winter, thereby possessing a lower stability efficacy in the rainiest season [13].

Biophysical properties can modify the contribution of roots in stabilizing slopes. Among Mediterranean trees and shrubs, for instance, Moresi et al. [14] found that the most resistant roots to breaking under tension were those of *Quercus cerris* L., while roots of *Ilex aquifolium* L. had the highest tensile strength among all shrub species. In cold or freezing environments, on the other hand, ornamental plants must be selected among those that are tolerant to low temperatures and evergreens. Analysing the plant-root-reinforced shallow slopes, Tsige et al. [15] observed that the effect of vegetation on slopes increased when the spacing between plants decreased, and that the slope angle modification in combination of plant roots had a relevant influence. Among the analysed species, *Salix subserrata* Willd. was the most promising plant species for slope stabilization, due to the effect of its better root mechanical properties.

However, it must be highlighted that the positive effects in the urban environment can be obtained if green space management is regularly carried out. Therefore, species must be selected considering technical parameters but also the municipal budget for urban green areas management [16].

Information related to the urban environment and to the ornamental plants used in the green areas is limited if compared to that of the natural environment, while it could be a support for city managers in taking decision about slope management. In the review, attention was therefore given to providing useful information for this purpose, with particular emphasis on the Mediterranean environment where extensive periods of water stress in summer and rainfall occurrences through intense precipitations that can turn into severe flash floods and floods can accentuate the problems of stability of the slopes.

1.1. Root Growth under Slopes Increase

Roots and canopies are strictly connected, and their relations dynamically change in terms of biomass, architecture, and organization during growth and according to the season conditions. Roots' distribution in soil follows the nutrients and water availability. Roots represent the transport system for nutrients and water and connect soil with leaves where the most part of metabolic processes take place. Each species has a specific roots architecture [17] and distribution in the soil (Figure 1). The genetic background of the species defines the potential root growth and distribution in the soil (i.e., depth, spreading, density) (Figure 1A–E). Roots mainly grow close and parallel to the soil surface and can strengthen the soil by intensifying the in-plane tensile strength of the rooted soil [5], while roots growing perpendicular to the soil surface strengthen the soil by improving shear-strength of the rooted soil mass on the sheared surface.

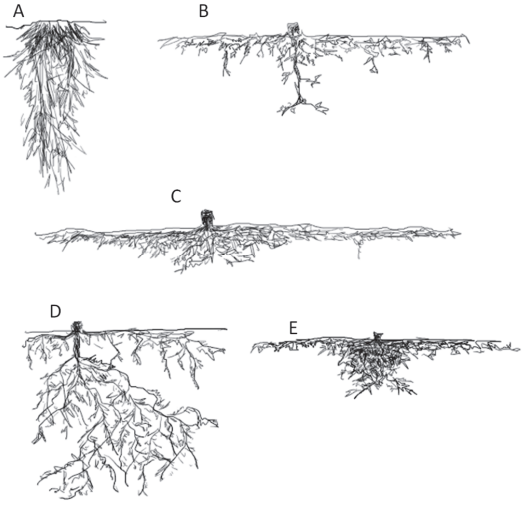

Figure 1. Different root systems development. (**A**) Tuft and deep root systems, (**B**) taproot root system, (**C**) superficial root system, (**D**) taproot and horizontal lateral roots, (**E**) heart-shaped root architecture. Redrawn by the authors from Ghestem et al. [5].

Roots of ornamental plants growing in slope conditions, in addition to nutrient and water transport, must guarantee plants' biomechanical stability and indirectly contribute to the slope stability. The increase of the slope induces different root responses and growth modifications. Mechanical stresses such as wind, rain, and gravitational force in sloping conditions influence root growth and their distribution, as mentioned above. As a consequence, the same species grown in different slope conditions can have different roots system that function to increase plant stability. Root growth under different slope degree has not been sufficiently detailed and this knowledge gap can represent limitations for plant species selection for practical applications. Further studies should be carried

out for elucidating how roots can balance the mechanical stability and their physiological functions.

Plant growth in slope conditions must follow the positive and negative gravitropic responses [18]. In a sloped soil, the aerial part and roots tend to align vertically during growth (Figure 2A). It means that plants in slope conditions have canopy and roots distribution that are not symmetric but this growth conditions could reduce the beneficial effects of plants for preventing landslides (Figure 2B).

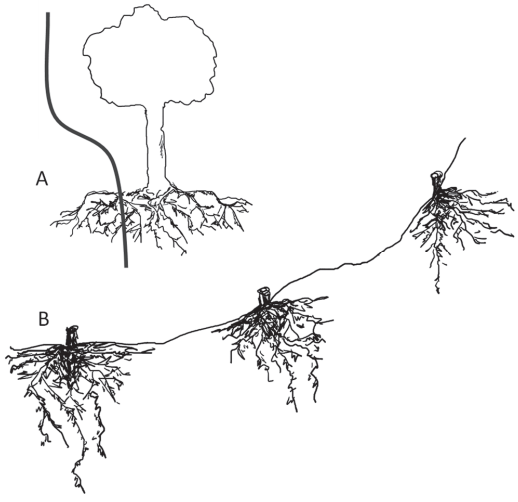

Figure 2. Plants grow in response to gravitropic stimuli: (**A**) the line represents the plant growth response to gravity. In slope conditions, the plants try to grow in a vertical position, reducing the angle between the ax of the trunk and the soil in slope, similarly, roots respond to gravity underground. Roots growth follows negative gravitropic response and aerial part positive response; (**B**) the increase of the slope influences in roots growth, development, and distribution which are a resultant of the gravitropic response and environmental conditions (original drawing).

As mentioned, root stability function is primarily determined by the biomass. However, root distribution in soil also plays an important role [19]. Unfortunately, the study of root distribution in soil is very complicated and in vivo monitoring of root growth and architecture distribution cannot be easily performed without having a perturbation effect on the roots system. On the contrary, invasive root analysis can lead to unreal results, especially regarding thinner apical roots. Plants located in slope conditions are more vulnerable to climatic events with potential danger for the closer buildings or roads [20]. Herbaceous plants combined with shrubs and trees can increase the roughness of the surface and enhance water infiltration, but higher water content in soil can increase the pressure and the weight and the susceptibility to landslides. The role of roots of ornamental plants in slope stability needs further investigations and multidisciplinary approaches are required for understanding the biological, hydraulic, and mechanical related aspects. Using biological solutions can have several applications for slope stabilization, but the lack of information regarding species behavior in different environments, especially in urban environments, represents one of the most significant limitations.

1.2. Root Morphology and Slope Stabilization

Ornamental plants should be selected considering the root morphology and development in relation to physical (sandy, clay, etc.) and chemical (mainly pH and salinity) soil proprieties and depth. For rocky slopes along urban areas with limited soil availability

ornamental plants with superficial (Figure 1C) or heart-shaped (Figure 1E) root systems should be used. On the contrary, for slopes with adequate soil depth plants with deeper root systems that can ensure higher slope stability can be selected [21]. The response of different root systems under different slope conditions should be further investigated for improving slopes stability through greening with ornamental plants.

2. Root Physiology and Development under Abiotic Stress

The root system is an integral plant organ involved in the acquisition of nutrients and water, the synthesis of plant hormones, organic acids, and amino acids, and it is necessary to ensure the anchorage of plants [22,23]. It also plays a fundamental role in maintaining cellular homeostasis under normal growth conditions and in plant-to-plant communication. Through the roots, plants absorb water and nutrients from the soil and transfer them to the aerial part. During stressful conditions, this equilibrium is modified, and the roots must implement structural and functional changes [24]. Root morphology and physiology are closely associated with the growth and development of aboveground plant material. However, it is known that the degree of the responses of roots to abiotic stresses may vary considerably within a family, a genus, and even a species [25].

In the presence of different abiotic stresses, the root system is modified due to presence of phytohormones that regulate this process [26]. The root is the initial part affected by abiotic stresses, and its morphological and physiological characteristics are closely correlated with plant resistance [27] or tolerance.

Different abiotic stresses affect the plants, and the drought stress is among the most important. In order to overcome drought stress conditions, plants modify the root system morphology and activate different physiological and biochemical processes [28,29].

A first response of the plant to an abiotic stress is the modification of the root biomass. In drought conditions, the roots, as the principal organ for water and nutrient uptake, play an important role in the plant drought tolerance [30]. *Penstemon barbatus* (Cav.) Roth, for instance, was able to tolerate drought by increasing root biomass and reducing stomatal conductance [31]. Similar results were observed in sunflowers (*Helianthus annuus* L.) [32] and *Catharanthus roseus* (L.) G.Don [33–35].

Even in presence of salt stress, the root biomass is modified. The osmotic stress caused by the salts present in the root environment induces a decrease in soil water potential at the root surface and, as a consequence, a difficulty for water uptake by the plants (decreases in leaf osmotic potential and leaf water potential) [36–38]. Fornes et al. [39] observed in three ornamental species (*Calceolaria* × *herbeohybrida* Voss, *Calendula officinalis* L., and *Petunia* hybr.) that root growth was reduced by salinity. During salt stress conditions, root dry biomass is an important parameter, because the higher root growth allows higher water and nutrient uptake to take place, favoring the accumulation of toxic ions in roots, in particular Na^+, thus diminishing its negative effects on shoot growth [40].

Drought and salt stresses can indirectly modulate root system architecture since they can produce unfavourable changes in the soil nutrient composition and distribution, soil density and compaction, and the type of soil particles [41]. Root growth is then deeply influenced by the availability and by the quality of water in the soil; the root system is the first to perceive the stress signs due to drought and salinity. Water scarcity inhibits the growth and development of the whole plant in numerous important species, while the root system, which is more tolerant than the aerial part, continues to grow even in the presence of low water potentials [42].

By increasing their root system, plants are able to explore the deeper layers to obtain water. An increase in the biomass ratio between roots and shoots (R/S ratio) under drought stress confirms this statement [43]. When the plant is affected by extreme soil drought, the regulation capacity, through asymmetric growth approach, may be also lost abruptly [43].

Due to drought stress, the plants modify the R/S ratio, for reducing water consumption and increase water absorption [44,45]. The increase in R/S ratio was observed in different species: *Lonicera implexa* Aiton [46–48], *Lupinus havardii* S.Watson [49], *Myrtus*

communis L. [50], *Nerium oleander* L. [51,52], *Opuntia ficus-indica* (L.) Mill. and *O. robusta* J.C. Wendl. [53], *Rhamnus alaternus* L. [54], *Rosmarinus officinalis* L. [55], two rose rootstocks (*Rosa multiflora* Thunb. and *R.* × *odorata* (hort. ex Andrews) Sweet) [56], *Sambucus mexicana* C.Presl ex DC. [57], *Silene vulgaris* (Moench) Garcke [58,59], in different shrubs of Mediterranean basin [60], and in *Viburnum tinus* L. [61]. Tribulato et al. [61] found an increase in root dry biomass (a higher R/S ratio) in *V. tinus* plants subjected to a severe water stress condition. This adaptation would allow to overcome the transplant condition and water shortage [62,63]. It is proved that the increasing R/S ratio is considered one of the avoidance mechanisms enabling plants to maximize the water uptake under drought stress condition [64].

In presence of drought stress, the plants need to maintain a greater root surface, while in salt stress conditions in some cases they can even reduce the root surface to limit the accumulation of toxic ions in the shoot, inducing in both situations a different distribution of the roots [65–67]. This can reduce water depletion around roots for minimising resistance to water transport to the root system [68], and modifies the water use efficiency that is improved, as demonstrated by Fernández et al. [69] on *Phillyrea angustifolia* L. Gomez-Bellot et al. [70] and by Álvarez and Sánchez-Blanco [71] who reported an increase in the R/S ratio in *Euonymus japonicus* Thunb. and *Callistemon citrinus* (Curtis) Skeels plants under moderate salt stress (EC 4 dS m^{-1}). Under salt stress condition this modification is frequently observed in plants [72]. Cirillo et al. [73] found unchanged R/S ratio in *Callistemon citrinus* and *Viburnum lucidum* L. plants subjected to salt stress; one interpretation of the unchanged R/S ratio may be a greater severity of NaCl stress (200 mmol NaCl).

During drought conditions not only the R/S ratio is modified but also other root characteristics such as root length, fresh weight (FW), dry weight (DW), diameter and surface area, deep rooting and cortex thickness and behaviours (i.e., root turnover, metacutisation, hardening, and hydraulic conductivity) can be influenced [68,74].

In *Callistemon* plants, Álvarez et al. [75] observed that water deficit increased the percentage of fine roots and decreased those with a diameter higher than 0.5 mm. In general, stressed plants showed a reduced root volume, although root dry weight was not modified, with the result that root density increased [34,75]. The root diameter increases with depth, and it is greatly linked to the uptake of water in deep soil layers [76].

Another factor that plays an important role in the tolerance of the drought stress is the hydrotropism [77,78]. Takahashi et al., [79] in a study about *Arabidopsis* and radish demonstrated that a gradient of moisture determined by water stress induces an immediate degradation of amyloplasts in the columella cells of plant roots, producing less response to gravity and increasing the hydrotropism.

A higher percentage of fine roots, able to penetrate the smallest soil pores, presumably optimises the exploratory abilities of the root system, and may play an important role for the survival of plants during drought stress [80]. Instead, in *Myrtus communis* L. and *Nerium oleander* L. plants, the percentage of thick roots increased, and the percentage of medium and fine roots was reduced following drought stress [50,51]. Different authors reported an increase in root diameter in different species (*Picea* sp., *Pinus banksiana* Lamb., *Portulaca oleracea* L.) in response to salt stress [74,81]. The higher robustness and accumulation of reserves, observed in these plants, could be linked to a higher root density [74,75,82].

Several studies have reported the association of the length, volume, and density of roots in crop species with drought tolerance [83–86]. Drought stress decreased the root length in *Abelmoschus esculentus* (L.) Moench [87], *Albizzia* seedlings [88], *Eucalyptus microtheca* F. Muell. seedlings [89], *Nerium oleander* L. [51], *Rhamnus alaternus* L. [67]. The opposite effects of drought stress on root length in other species agreed with the results of Chyliński et al. [90] on geranium (*Pelargonium hortorum* L. H. Bailey) and impatiens (*Impatiens walleriana* Hook) also reported by Shober et al. [91] on *Viburnum odoratissimum* Ker Gawl. and by Franco et al., [59] on *Silene vulgaris* (Moench) Garcke.

The development of lateral roots is inhibited in conditions of water stress, while the induction of new roots does not change and the development of primary roots increases

due to the hydrotropism that are under the control of ABA [92]. However, this is not always confirmed. Indeed, in a study conducted by He et al. [93] on *Camellia oleifera* Abel, subject to drought stress, plants at the end of the experiment did not show a significantly decrease in the number of lateral roots. The root-crown and root-plant ratios indicated that in drought conditions the plant gives priority to the normal development of the root system, by maintaining the contact area with the soil, thus obtaining the necessary water [94]. This has also been observed in other studies [95].

Hardening of roots, measured by increasing of brown root percentage, is frequent in drought-stressed plants [68]. Plants of *Limonium cossonianum* Kuntze [96], *Lotus creticus* L. [47] and *Silene vulgaris* (Moench) Garcke plants [59], with limited water availability, showed variation of root colour, from white to brown, that is linked to the suberisation of the exodermins and it is an index of metacutisation process. This was also found in *Rosmarinus officinalis* L. by Sánchez-Blanco et al. [55] and in *Nerium oleander* L. by Bañón et al. [51]. In two trials conducted by Franco et al. [59] about *Silene vulgaris* subjected to drought stress an increase in cortex thickness:root radius (C:R) ratio was observed improving the resistance to dehydration. These changes could increase the resistance of *Silene vulgaris* seedlings at the drought conditions.

On the contrary, flooding or waterlogging represent extreme conditions for plants and roots as the first target of waterlogging stress in plants. In urban environments, severe soil compactions and limited drainage can induce waterlogging, thus hypoxia and anoxia effects on plants. Soil waterlogging has in fact been identified as one of the main abiotic stresses in urban areas; the constraints imposed on the root have marked effects on the growth and development of plants [97,98]. Waterlogging inhibits respiration in the root, due to an insufficient supply of oxygen [99]. Hypoxia is the main stress factor in waterlogging conditions [100] and the primary effect of soil flooding is to slow down the oxygen transfer to the roots. This limits their aerobic respiration and dramatically depresses their metabolism. In addition, if tissues are hypoxic, the aerobic energy is reduced and the functional relationships between roots and shoots are compromised [101,102].

The development of adventitious roots is stimulated by the increase of ethylene production in the shoots of the trees or for effect of external increasing of the compound in the soil solution [103]. Formation of adventitious roots in response to ethylene has been considered a major adaptive mechanism of wetland plants to root damage caused by waterlogging stress [104]. Adventitious roots emerge and grow horizontally close to the water surface, and they are connected to the stem close to the site of aerenchyma formation [105]. Hence, adventitious roots can facilitate oxygen capture of submerged tissues alleviating the hypoxic conditions and contributing to the recovery and maintenance of aerobic respiration in waterlogged seedlings [106,107].

Under salt stress conditions, the root mitochondrial electron transport might be disrupted, promoting O_2 accumulation in a manner similar to that from hypoxia conditions [108]. Tolerant species exhibit several physiological and biochemical modifications including quasi-dormancy of shoot tissues, stomatal closure, elongation of submerged stems, and the formation of aerenchyma in existing root tissue or development of new nodal roots at the stem base [101].

The density of the xylematic vessels is one indicator of plant capacity to absorb and transport water in the roots. In drought conditions, a higher vessel density could increase the tolerance. This was observed in a study on *Lotus creticus* L. subjected to drought stress [48].

In northern countries, the abiotic stress predominant during winter is due to cold or freezing. The cold can damage the cell membrane and the severity of damage can reach the leaves or branches of evergreens. In slope conditions, the plants used for enhancing the stability must have a high tolerance to freezing temperatures, especially at the root level. The ice formation in the soil can induce roots damage or death. In these conditions, landslides can occur with the de-icing when the connection between roots and soil is

lessened. In cold environments, it is very important to select winter herbaceous species and evergreen shrubs or trees.

As above described, certain abiotic stresses could stimulate root development and indirectly improve slope stability. The green areas of slopes are not subjected to constant management, and therefore the abiotic stresses such as drought represent common and frequent stress in peri-urban greens. Based on the predominant abiotic stress, suitable ornamental plants should be selected so that the response of the plants will probably be an increase of the root systems that improves the soil stability.

3. Use of Plant Growth Regulators and Biostimulants for Increasing Root Biomass

In slope conditions, transplanted plants rapidly need to develop their roots to ensure a good anchorage with soil so that they can soon reach stability. The root development can be enhanced with plant growth regulators such as cytokinins, auxins, or using plant biostimulants. The increase of roots length and biomass can be a response of a plant hormones equilibrium that is influenced by the external stimuli. Beside plant growth regulators, biostimulants can be also used as agronomic tools for stimulating root development and biomass accumulation. Research on biostimulants and roots demonstrated that several of these products can be effective for root formation and growth. Biostimulants can be derived from different raw materials, seaweeds, plant extracts (botanicals), inorganic compounds, beneficial fungi, and bacteria and are commonly used for increasing plant growth and abiotic stress tolerance [109,110]. Biostimulant applications in nursery or after transplant can enhance root formation.

The main application of these products is for production purpose in horticultural crops, but also in urban environments [111]. However, their environmental impact is low since most of them have organic nature. Among the plant extract, there are only few published works. Willow bark extract formation was effective in the development of adventitious roots and root branching in lavender and chrysanthemum [112]. Plant growth-promoting rhizobacteria (PGPR) applications can also improve root biomass and functionality and several positive results have been reported for flower and ornamental plants [113]. In *Eucalyptus* clones (hybrid *Eucalyptus grandis* W. Hill × *E. urophylla* S.T. Blake) the application of *Aspergillus flavipes* (ATCC®16814™) has been used as a novel biostimulant for rooting-enhancement, in terms of biomass and root length [114]. The positive action of PGPR is also due to the induction of auxins or cytokinins by the roots. The fast root development after transplant can rapidly cover the soil and in slope conditions is very important for reduce erosion.

The nursery phase influences plant development after transplant and also the root systems. In *Lotus creticus* L. subsp. *cytisoides* (L.) Arcang., the irrigation two days/week instead of six days/week determined a greater root length: shoot length ratio and a higher percentage of brown roots more favourable to tolerate transplant stress [115]. Also, treatments with arbuscular mycorrhizal (AM) in the nursery phase can improve the root system architecture and resistance to drought in *Pistacia lentiscus* L. [116].

Biostimulant fungi based such as *Trichoderma* increase root growth and nutrient uptake by the induction of auxin biosynthesis [117]. In *Impatiens walleriana* Hook. f. plants treated with *Trichoderma* isolates showed longer roots and higher roots dry weight than control and comparable with commercial indole-3 butyric acid (IBA). These studies demonstrated that different isolates have different efficacy and appropriate species should be used for roots induction and development [118]. Mexican petunia (*Ruellia brittoniana* Leonard) treated with humic acids, amino acids, and active dry yeast showed an increase in root length and weight, indicating the potential role of these compounds in roots development [119].

Seaweed extracts obtained from *Ascophyllum nodosum* (L.) Le Jolis applied to *Passiflora actinia* Hook. increased 10% of rooting with 40% seaweed extract [120]. The rooting stimulation of a commercial biostimulant-based seaweed extracts has been also demonstrated in woody cuttings of *Camellia japonica* L. [121]. Analogous results were also observed cutting of old rose cultivars [122,123]. These results were observed at nursery using cuttings; pre-

or post-transplant application should be also studied to understand if biostimulants could be applied as application for inducing rapid root development and reaching a rapid vegetation covering of slopes. Moreover, biostimulants could be also applied during the green management on slopes. These products stimulating the root development can rapidly enforce the integration of vegetation with soil and increase the slope stability.

4. Use of Ornamental Plants for Slope Greening

Ornamental plants are often valued only for their visual aspect. For this reason, the concept of ornamental plants is frequently used in its broadest sense to include plants that are grown for decorative purposes such as in the case of gardens, home gardens, landscape design projects, squares, parks, street trees, indoor plants, and cut flowers [124]. Recently, very widespread ecological requests have determined that ornamental plants are not only beautiful, but that any plant able to improve the environment and the quality of our lives [125] by providing ecosystem services is valuable. Ornamental plants can be adopted to restore degraded landscapes, and in particular to control erosion. In consideration of the numerous green area typologies and the breadth of the meaning of 'ornamental', the number of species that can be used is extensive [29]. The wide number of ornamental or potentially ornamental species enhances the possibility of identifying genotypes that are able to cope with the different conditions where these plants can be used.

However, it is also important to evaluate the geographical distribution of the different plant species during selection. It is advisable to avoid exotic species that can cause invasiveness problems. Soil erosion is a typical environmental problem that inflicts numerous and serious damages in agricultural cultivations as well as in natural ecosystems. In particular, erosion reduces the water-holding capacity of plants due to rapid water runoff and reduces soil organic matter [12], which can also affect green areas (especially those realized in slope surfaces). The key role of plant cover in controlling water erosion is widely accepted. According to Naylor et al. [126], the effects of vegetation on soil can be divided into two major categories: bioprotection, by reducing water runoff [12], and bioconstruction, by increasing water infiltration into the soil matrix [127].

Plants with their roots fix the soil [128] and with their canopy reduce the energy of raindrops [12]. The way the plants are arranged along the slopes can decrease the sediment runoff [129] (resulting from superficial down slope transport of soil particles) [7,130].

Gyssels et al. [130] stated that the aboveground vegetative cover was the most important factor to splash and interrill erosion processes; the roots were as important as aboveground vegetation cover for rill and gully erosion processes. The relative contribution of roots to runoff and soil loss reduction varied with vegetation types. Roots conserve soil or increasing infiltration, thus reducing runoff and soil loss [131], or improving soil properties by increasing soil organic matter levels, enhancing the quantity of soil stable aggregates and stabilizing soil layer structures [132,133].

Unfortunately, information on root characteristics of ornamental plants and their effects on the topsoil resistance to concentrated flow erosion is lacking. Roots influence the properties of the soil, such as infiltration rate, aggregate stability, moisture content, shear strength, and organic matter content, all of which control soil erosion rates to various degrees. The presence of roots also increases soil roughness, improving the capacity for water infiltration and reducing surface runoff velocity [134].

The impact of herbaceous and woody plants on soil erosion is crucial and different according to the species. Since ornamental plants can be both herbaceous and woody, it is possible to count on both effects against soil erosion. Perennial grasses provide year-round soil cover and reduce water runoff and sediment loss and promote soil-development processes by enhancing soil organic matter, soil structure and soil water and nutrient-holding capacity. Dense root architecture and vegetative cover on soil surface can reduce soil erosion. Woody plants reduce water erosion by improving water infiltration, reducing the negative effects of droplets, intercepting rain and snow and stabilizing the soil through roots and leaves.

In the semi-arid Mediterranean region, where water erosion is particularly severe, different experimental studies on the influence of the native vegetation on erosion have quantified soil loss and runoff under woodlands or shrublands comprising a mixture of plant species [135–137]. All of these studies have shown that typical Mediterranean shrubland vegetation is very efficient in reducing water erosion, also under extreme torrential simulated rainfalls [138].

In general, under similar climatic and topographic conditions, shrubs are most efficient in reducing runoff and sediment levels, followed by herbaceous plants and trees [133]. The coverage rate plays a similarly important role as vegetation type in affecting runoff and soil loss; the effects of vegetation types and coverage rates on runoff and soil loss are related.

Not all grass species, despite their many fine roots, appeared to have strong roots [139], and so species choice is very important in reducing erosion. The choice of suitable plant species depends on the context. De Baets et al. [140] used four criteria to evaluate the capacity of different species to control erosion, i.e., plants having: (i) a high potential to prevent incision by concentrated flow erosion, (ii) the potential to improve slope stability, (iii) the potential to resist bending by water flow, and (iv) the ability to trap sediments and organic debris. The scores for these indicators were represented on amoeba diagrams, indicating the strengths and the weaknesses of plant traits, in relation to erosion control. The scoring of plants on these criteria was based on a multi-criteria analysis. In the species choice, the plant tolerance to abiotic stress, and in particular to drought gains relevance, especially in the Mediterranean area.

In an experiment Bochet et al. [141] analysed the relative efficiency in reducing water erosion on slopes of three representative species of the Mediterranean vegetation, that showed different plant morphologies (*Rosmarinus officinalis* L., *Stipa tenacissima* L., and *Anthyllis cytisoides* L.). The results showed that the three species differently reduced runoff and soil loss. *Stipa* plants, characterized by dense canopy, counteracted rainfall erosivity, reducing splash erosion. *Rosmarinus*, in addition to the canopy effects, improves the topsoil structure by means of incorporation of organic matter. The litter cover seems to be very important in erosion control. *Anthyllis*, that is a deciduous shrub, give little protection against the impact of rain on soil surface as compared to a bare surface.

Burylo et al. [142] carried out an experiment to investigate the effect of the root systems of three species [*Robinia pseudoacacia* L. (tree), *Pinus nigra* var. *austriaca* (Höss) Badoux (tree) and *Achnatherum calamagrostis* (L.) P. Beauv. (grass)], on concentrated flow erosion rates. Ten functional traits, related to plant morphological and biomechanical features, were measured. Analyses were conducted to identify traits that cause plant root effects on erosion control. Erosion rates were lowest for samples of *R. pseudoacacia*, intermediate in *A. calamagrostis* and highest in *P. nigra* var. *austriaca*. The study also highlighted the role of fine roots in reducing erosion rates.

To compare the contribution in erosion control, five fern natives of southern China, namely, *Blechnum orientale* L., *Cyclosorus parasiticus* (L.) Farw., *Dicranopteris pedata* (Houtt.) Nakaike, *Nephrolepis auriculata* Trimen, and *Pteris vittata* L., were selected. The leaf area index, root area ratio and root density were significantly correlated with erosion-reducing potential. Among the species, *N. auriculata* performed better the other species by showing higher values of the determined plant traits [143].

Over the species choice (Table 1), the relationships between vegetation structural attributes (spatial pattern, functional diversity), soil surface properties (crust, stone, plant, and ground cover, and particle size distribution) and hillslope hydrologic functioning have been kept in consideration [144]. Since a typical landscape is a blend of species (herbs, grasses, shrubs, trees, etc.), it is possible to organize the plant arrangement to obtain the best result in erosion control to take advantage of plant and root characteristics.

Table 1. Species with ornamental value [1] suitable for erosion control.

Species	Family	Plant Habitus	References
Amorpha fruticosa L.	Leguminosae	Shrub	[145]
Anthyllis cytisoides L.	Leguminosae	Shrub	[128,139,140,146]
Artemisia vulgaris L.	Compositae	Herb	[146]
Atriplex halimus L.	Amaranthaceae	Shrub	[128,139,140,147]
Carpobrotus edulis (L.) N.E.Br.	Aizoaceae	Succulent	[148]
Comptonia peregrina (L.) J. M. Coult.	Myricaceae	Shrub	[149]
Dorycnium pentaphyllum Scop.	Leguminosae	Shrub	[128,146]
Hedera helix L.	Araliaceae	Climber	[148]
Hippophae rhamnoides L.	Rhamnaceae	Shrub	[150]
Lantana montevidensis (Spreng.) Briq.	Verbenaceae	Shrub	[148]
Lavandula lanata L.	Lamiaceae	Shrub	[7]
Limonium supinum (Girard) Pignatti	Plumbaginaceae	Herb	[139,140]
Lonicera japonica Thunb. 'Repens'	Caprifoliaceae	Climber	[148]
Medicago arborea L.	Leguminosae	Shrub	[151]
Myoporum parvifolium R. Br. 'Prostratus'	Scrophulariaceae	Shrub	[148]
Nephrolepis auriculata (L.) Trimen	Nephrolepidaceae	Fern	[143]
Nerium oleander L.	Apocynaceae	Shrub	[128,140,146]
Opuntia ficus-indica (L.) Miller f. *amyclaea* and f. *elongata*	Cactaceae	Succulent	[152]
Origanum bastetanum L.	Lamiaceae	Herb	[7]
Origanum vulgare L.	Lamiaceae	Herb	[147]
Psolarea bituminosa L.	Leguminosae	Herb	[151]
Putoria calabrica (L.) DC.	Rubiaceae	Shrublet	[153]
Retama shaerocarpa (L.) Boiss	Leguminosae	Shrub	[128,139,140,146]
Robinia pseudoacacia L.	Leguminosae	Tree	[142]
Rosa abyssinica R. Br. ex Lindl.	Rosaceae	Shrub	[153]
Rosmarinus officinalis L.	Lamiaceae	Shrub	[128,140,141,146,153,154]
Rosmarinus officinalis L. 'Prostratus'	Lamiaceae	Shrub	[148]
Salsola genistoides Juss. ex Poir.	Amaranthaceae	Shrub	[128,139,140,146]
Salvia lavandulifolia Vahl	Lamiaceae	Shrub	[7]
Santolina rosmarinifolia L.	Compositae	Shrub	[7]
Senecio jacobaea L.	Compositae	Herb	[147]
Tamarix canariensis Willd.	Tamaricaceae	Tree	[128,139,140,146]
Tanacetum vulgare L.	Compositae	Herb	[147]
Tephrosia vogelii Hook. f.	Leguminosae	Tree	[155]
Vinca major L.	Apocynaceae	Herb	[148]

[1] Species of Poaceae family, often successfully used for erosion control, are not considered.

Berendse et al. [156], analysing the effect of the use of four diversity treatments (one, two, four, and eight species commonly found in grassland), found that plant species diversity has important effects on the erosion resistance of slopes. The loss of species diversity, in fact, reduces the erosion resistance. They concluded that the presence of diverse plant communities on slopes are essential to minimize soil erosion.

In the US, highway departments request wildflowers for erosion control. On roadsides, wildflowers ensured a source of colour as well as erosion control [157]. Effects of wildflowers are linked to their biodiversity that helps to reduce soil erosion [158]. Roads have a great impact on the environment, habitat fragmentation, soil erosion, edge effects, and pollution. In order to reduce such impacts, native plants, naturally occurring in roadside vegetation and well adapted to those conditions, provide highly effective mixes for revegetation [159–161].

García-Fayos and Bochet [161], analysing climate change consequences and the increase in soil erosion, found that high plant species biodiversity and plant cover are negatively influenced by climate change and soil erosion, which negatively influences soil resistance to erosion, nutrient content, and water holding capacity. They also reported that plant species diversity weakly correlates with plant cover, but strongly with soil characteristics related to fertility, water holding capacity, and resistance to erosion.

In the experiments, different species were analysed to identify important plant traits that influence the hydraulic roughness to contrast erosion. The results indicated the stronger effect of density-weighted traits, demonstrating that communities with the best trade-offs among stem density, diameter, and leaf area are the key to mitigate soil erosion. For these reason, herbaceous ecosystems could play an important role in soil erosion mitigation [147]. Herbaceous vegetation, in fact, was more efficient than trees in improving aggregate stability [162]. In crop trees of *Vernicia fordii* (Hemsl.) Airy Shaw, aggregate stability was improved in the presence of herbaceous *Artemisia codonocephala* Diels. Mixtures of different plant functional types, typical of landscape arrangement, would improve soil conservation on slopes, by reducing both surface water erosion and shallow substrate mass movement [162]. The combination with nitrogen-fixing species will also be useful for providing this element for improving roots growth and development.

5. Limitations and Research Gaps in the Use of Ornamental Plants for Improving Slope Stability

The use of plants represents a long-term solution for preventing landslides in slope soils. Nevertheless, there can be some limitations that can be summarized as follows:

- *Slow roots establishment and stability*: the contribution of roots to stability increases with the roots' development and establishment. The highest stability is achieved when the species used reach maturity and the roots network is well integrated with the soil. Therefore, the stability of the slope is not immediately obtained (a limitation to be considered);
- *Unpredictable environmental effects*: plants development depends on environmental parameters and unpredicted events can reduce the efficacy of plants used in slope stabilization. These can also include soil-borne diseases and the limitation of the use of specific agrochemicals can represent an important limit in the maintenance of plants health.
- *High costs and regularity of maintenance*: public urban areas are under municipality maintenance and the lack of funds or of regularity in the management can compromise the benefit of the plants on the stability of the slopes;
- *Minimum of soil for plants growth*: the use of plants as slope stabilizers can be a solution if there is a minimum of soil for roots development and for a better anchorage that can harness the soil itself avoiding landslides.

The research gaps in the use of ornamental species for slope stabilization are represented by the limited case studies. Research activities should be focused from plant material propagation to agronomic management in the early stage of plant growth after transplant.

At the nursery level, appropriate strategies should be adopted to increase the biomass and functionality of the roots to enhance their development after transplants. Agronomic strategies should be evaluated for increasing roots development using PGPR or biostimulants. A wide range of species combinations should be studied in different urban environments with different soil and different slopes. In particular, further studies are required for evaluating the effect of abiotic stresses on the ability of plants to prevent landslides. Limited information is available for the effect of cold or freezing temperature on roots metabolism and their potential roles as slope stabilizers.

Moreover, the stability of vegetated areas should be mechanically determined for providing objective results. Since plants are living organisms, the stability of slopes should be studied over a long period and the weakness associated with the age of the vegetated area should be identified.

6. Conclusions and Outlook

Ornamental plants in urban and peri-urban areas have a wide range of beneficial effects that are beyond the aesthetical values of green areas. The positive role of ornamental plants on urban environments and their effect on the residents is well known. This review

provides information and suggestions concerning the utilization of ornamental species, including herbaceous and woody species, as biological elements that can be exploited for improving the stability of slopes and reducing the risk of landslides. The potential benefits of ornamental plants have been illustrated and the biological traits that could help in improving slope stability have been reported and discussed. In this respect the recent events in Western Europe and North Italy, in regards to flooding and landslides, have shown that slope management is also of paramount importance in countries where these kinds of events used to be rare in the past but are becoming more frequent due to climate change. Under this scenario the application of nature-based and hybrid solution (i.e., nature-based solutions in combination with conventional engineering solutions) approaches for landslide risk management is calling for the attention of researchers. One of the major questions to be answered regards the selection of suitable species. Species with deeper roots are more effective in preventing shallow soil failures, as their roots and stems provide mechanical reinforcement and restraint and their root uptake and foliage interception modify slope hydrology. The length of the establishment period is also important in selecting species since some species have been shown to establish themselves better and faster than others. Further, with regard to woody species in particular, information on root system architecture, root growth rates, and lifespan would provide environmental managers with data which would enable them to more efficiently manage slopes. Unfortunately (and mainly due to the difficulty of carrying out in situ researches that are time and money consuming and not easily replicable), most of this information is not still available, and therefore further investigations are required for providing enough details that could help the ornamental species selection for slope condition areas.

From the analysis of the literature, a need to extend research to other plant species, differing in root architecture, and to comprehend how future results can be applied at practical level is emerging. Although many models have been developed to predict rainfall dynamics and subsequent erosion potential, few of these have taken root architecture and dynamics into account in terms of their ability to improve soil water flow predictions. This review provides useful suggestions and research directions that can be considered for further studies and investigations focusing on ornamental plants as key elements for controlling erosion and increasing slope stability.

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Review

Can the Caper (*Capparis spinosa* L.) Still Be Considered a Difficult-to-Propagate Crop?

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Abstract: As a perennial xerophytic shrub, characterized by plesiomorphic features, the caper (*Capparis spinosa* L.) is naturally spread throughout the Mediterranean basin and occupies an important ecological role, as well as an economic one, in traditional and specialized systems for commercial production. This species, in spite of its wide diffusion, is currently considered at risk of genetic erosion, mainly due to overgrazing and overharvesting for domestic uses and for trade. This situation is made more serious because of the lack of efficient propagation techniques, determining the caper as a "difficult-to-propagate species". In this review, we report the main available sexual and vegetative propagation techniques with the aim of assessing whether, and to what extent, this criticality is still true for caper as a horticultural crop. In terms of seed propagation, germination rates have generally been considered quite low or unsatisfactory, and are also affected by hybridization phenomena that are likely to occur among both the wild and cultivated forms. The seeds show a physiological dormancy that can be lowered by adopting hormonal treatments, but in situ germination remains a critical phase. Vegetative propagation appears quite effective, mostly as related to in vitro techniques that allow caper cultivation that is no longer affected by propagation for an economic dissemination of the species in more intensive orchards. The research needs for *Caper spinosa* L. as a horticultural crop, especially in the field of genetic improvement and breeding, are also underlined.

Keywords: *Capparis spinosa*; seed propagation; vegetative propagation; in vitro propagation; Mediterranean basin

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1. Introduction

The caper (*Capparis spinosa* L.) is one of about 250 species of the genus of xerotropical origin belonging to the family Capparaceae, widely distributed from the Mediterranean eastwards to Central and Southeast Asia, Australia and Oceania [1]. This perennial xerophytic shrub, characterized by plesiomorphic features, is naturally spread throughout the Mediterranean basin, composing part of the Mediterranean Maquis together with other species [2–4], participating with two subspecies, *C. spinosa* subsp. spinosa and *C. spinosa* subsp. rupestris [5]. It is also widely diffused in cultivated form, especially in Southern Europe (Italy, Spain and Greece), in North Africa (Tunisia, Morocco and Egypt) and in the Middle East (Syria and Turkey), in traditional and specialized systems for commercial production of pickled, in salt or vinegar, immature flower buds (the caper of commerce), unripe fruits (caper berries) and tender shoots, or as an ornament. The capers for commerce and the other related products have been used since ancient times [6] for food or as a condiment [7,8], as well as in ethnopharmacology, medicine and cosmetics, due to a number of bioactive compounds with beneficial properties and effects [9–15]. However, most of the caper products available in the international market rely largely on the wild plants, as

cultivation is not able to meet the thriving market demand [6]. In Italy, caper cultivation, based upon local selections or biotypes [16] of *C. spinosa* L., domesticated in situ from subsp. rupestris [17], is concentrated in the small islands around Sicily (mostly Pantelleria and the Aeolian Islands Salina, Lipari and Filicudi). In these Sicilian areas, the species plays a significant socio-economical role [18,19] and was awarded in 1996 with the designation of UE "Protected Geographical Indication" (PGI) for the caper grown on the island of Pantelleria (Figure 1), and more recently (2020), for that on the Aeolian archipelago, by EU "Protected Designation of Origin" (PDO-IT-20A02570), under the denomination of "Cappero delle Isole Eolie Dop". On the Aeolian island of Salina (Figure 2), a Slow Food Presidium has been active for many years, with the aim of preserving a heritage of cultural and agronomic knowledge linked to the island's history and the resilience of its heroic agriculture [20].

Figure 1. Traditional caper orchard on the island of Pantelleria, where sloped lands in ancient times were managed in order to obtain comfortable terraces.

Figure 2. Intensive caper orchard on the island of Salina.

Well adapted to harsh environmental conditions [21], due to its xerophytic habit and its deep root system [22,23], its exceptional photosynthetic characteristics, maintained under high irradiance and temperature without damage symptoms [24], and its adaptation to poor soils [25], *C. spinosa* L., could, therefore, significantly contribute to the development of local, marginal areas [21], especially those of small islands. Additionally, caper may help preserve the equilibrium of fragile arid and semiarid ecosystems [26] by reducing the soil erosion

hazard [27], by enhancing landscape sustainability, water conservation [28], rehabilitation of rangelands and desert lands and dune stabilization [29,30] and by preventing fires from spreading [31]. Altogether, these features make this species of potential strategic interest as an adaptive crop in a climate change context [32].

Nevertheless, despite its natural diffusion and cultivation, *Capparis spinosa* L., as a Wild Harvested Plant (WHP), is currently considered at risk of genetic erosion, mainly due to overgrazing and overharvesting for domestic uses and for trade. In fact, it has been included in the IUCN Red List of Threatened Species [33], even if assessed globally as "Least Concern", and as an endangered species, among the Neglected and Underutilized plant Species (NUS) [19,34].

One of the major constraints, both in the cultivation and in the conservation of WHP or NUS species in the Mediterranean region, is generally represented by the lack of modern, adequate and efficient propagation techniques [35]. Caper has long been considered as a "difficult-to-propagate species", mostly because of the poor seed germination rate generally observed [36], together with the heterogenous population obtained from seed [8,29] and the frequent erratic response of rooting [37,38].

The question that will be discussed in the present review is, therefore, whether, and to what extent, this reputation is still true for caper as a horticultural crop. Here, we describe the main available propagation techniques and the main factors influencing the results by reviewing the most relevant literature regarding both sexual and vegetative caper propagation. The underlying hypothesis is that the caper industry is, from an agronomic point of view, still in its infancy and that, therefore, some of the "traditional" propagation systems still widely utilized may be no longer valid for modern and specialized caper cultivation.

2. Seed Propagation

Caper propagation in natural habits is ensured by seed dispersal exerted by several animals attracted by the juicy flesh of caperberries, such as lizards, ants, wasps, birds, rats and rabbits [39–42] which transport and release the undigested seeds, thus enhancing the permeability of the seed coats, and ultimately increasing the seed germination rate. These plants obtained from seed exhibit a high degree of heterogeneity in morphophysiological traits that are conducive to species adaptation and resilience in wild habitats, and may be of extreme interest for breeding purposes, but decidedly not for the agronomic exigence of homogeneity. Nevertheless, according to Chedraoui [21], seed germination is the method of propagation most utilized for caper plant cultivation, and some authors even report [18] that these seedling plants are considered by the farmers "homogeneous enough for the purposes of quality and quantity requirements of the crop". As a matter of fact, seed propagation, also favored by the caper seedlings' short juvenile period and the high number of seeds contained in each fruit (up to 400), is still commonly used in Pantelleria for caper cultivation, although using some farmers' selected lines [16,38], as well as in many other countries. Furthermore, it should be noted that caper seeds, if well stored (i.e., 4–7 °C and low relative humidity), can maintain their germination potential for about 2 years [43,44] or even more than 3 years [45,46] before gradually decreasing.

The seed propagation techniques used in Pantelleria [38] include elemental operations and are carried out in small seedbeds arranged outdoors, without either any disinfection or pre-germination seed treatment or regular irrigation. A good part of the seed population germinates 25–50 days after sowing; however, it is not uncommon to record germination after one or two years. Seedling transplanting is performed during the following winter.

On the other hand, in Spain (Figure 3), nursery seed propagation includes seed stratification in sand, followed by sowing in raised beds in the second half of April to the beginning of May, using 1.5 or 2 g of pre-germinated seeds per linear meter, corresponding to approximately 250–330 seeds m^{-1} [37]. Seeds lots are collected in August–September from ripe, opened fruits, when flesh color is dark red and seed color is dark brown. They are then rinsed with water, separated from the flesh, and gently dried in the shade before stratification to soften the seed covering. This is carried out after a fungicide treatment in

stratified, opportunely moistened sand layers, each 2–4 cm high. This process takes 25 to 50 days and generally leads to a 30–40% pre-germination rate. The full emergence of the plantlets (45–50 out of the initial 250–330 pre-germinated seeds) occurs 25–30 days after sowing. Simplified direct seeding with 4–5 g seed m^{-2} is also reported, but with lower results in terms of plantlet emergence, with the best expectations of 30–40 plantlets m^{-2}.

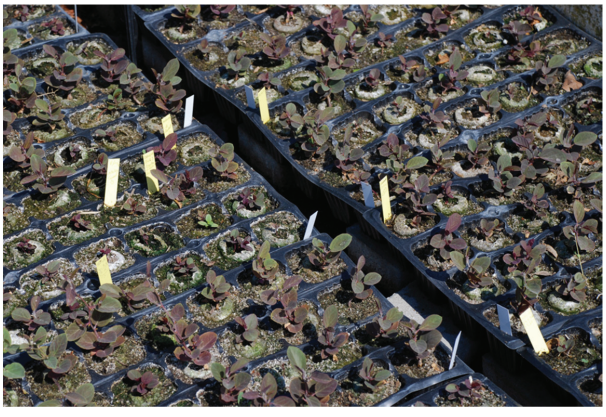

Figure 3. Experiments in seed propagation carried out in Spain at CITA by using seed of capers from Ballobar (Aragon, Spain).

Variable germination rates, considered generally low or unsatisfactory, are reported in the scientific literature [18,36,43,47,48]. Most of the unsuccessful results have been almost unanimously related to morphological and physiological seed characteristics such as the thick, lignified structures of the seed integuments [44] and the mucilage surrounding the seeds, developed in contact with excessive water, which represents an effective barrier against the diffusion of oxygen to the embryo [43]. However, recently it was demonstrated that this barrier can be easily removed through seed leaching for 12 h [49].

Additionally, it must be considered that the poorer germination percentages are reported mainly after direct sowing into the field [26], where disturbing factors, such as predator activity, inter alia, cannot be excluded. Several other factors can also affect the final germination percentage such as seed viability, degree of fruit ripening, fruit position on the plant and fruit weight [50]. Furthermore, the extreme heterogeneity of the results present in the scientific literature so far is an indication of the inherent genetic variability of the utilized seed lots, as affected by seed provenance but also by hybridization phenomena that are likely to occur among both the wild and cultivated forms of *C. spinosa* L. [5,16,17,51–53].

At the same time, an effect on germination exerted by cross-pollination between different ecotypes or accessions of *C. spinosa* cannot be excluded. However, to the best of our knowledge, no specific data on such an issue are available in the scientific literature and, therefore, it may be of interest to be studied also for breeding purposes. Different techniques can be used to improve the germination performance of woody perennials [54]. These consist of treatments to overcome embryo dormancy and/or physical–mechanical dormancy. The latter is generally removed through the effects on the seed covering by stratification (softening) or scarification (scratching). Embryo dormancy, a physiological status under hormonal control, can be overcome by seed vernalization in outdoor beds during winter or in climatic chambers and/or by the application of various chemicals and plant growth regulators (PGRs) such as gibberellins (GA), cytokinins and other substances. Most of these possible treatments, with variable results, have been tested on *C. spinosa* L., and have been partly reviewed by Sozzi and co-workers [36]: mechanical scarification, stratification, soaking in hot water (55–85 °C), concentrated H_2SO_4 or H_2O_2, or in 0.2% $KMnO_4$, 0.2% KNO_3, gibberellin (GA_{4+7}) or gibberellic acid (GA_3) aqueous solutions, and

by manipulation of the environmental conditions (light/dark, temperature). A significant advantage (100% viable embryos germinated within 3–4 days) was obtained by partially removing the seed coats from non-germinated seeds [44]; thus, the authors underlined the supposed inhibitory role exerted by seed integuments. The combined effect of ultrasonic wave treatments (1700 KHz), applied in the presence of GA_3, proved able to increase the germination percentage [55]. In an experiment carried out in vitro, high seed germination percentage was achieved on MS medium deprived of hormones (71%) and on sterile water (64%) after a dormancy-breaking treatment by coat scarification [56]. Cold stratification showed positive effects on germination percentage and rate [22,37].

In a study performed on the effects of soaking period and GA_3 addition [57], it was concluded that adding a GA solution to the substrate, after acid scarification, was a simple and effective method to ensure satisfactory caper seed germination, although a possible negative effect of acid scarification on successive seedling growth has been reported, together with negative effects of different levels of salinity on germination [58]. In another experiment, a soaking period of 30 days or longer, with or without the addition of a GA_3 solution to the substrate, proved to enhance seed germination speed, duration and percentage (up to 95–99%) [59].

In a study aimed at the evaluation of caper seed germination, under interaction of different concentrated sulfuric acid (CSA) and GA_3 concentrations, it was found that CSA for 30 min and 200, 300 or 400 mg L^{-1} GA_3 solutions led to the highest (~60%) germination percentages [29], leading the authors to underline the greater opportunity to combine both scarification and GA_3 treatments. Similar results (62% seed germination) were obtained in India using sulfuric acid (40 min), followed by dipping in 400 ppm GA_3 for 2 h [60] using *C. spinosa* seeds harvested in the wild, cold desert of the Ladakh region and in Turkey, with seeds initially immersed in warm water (40 °C) and then treated with H_2SO_4 for 20 min and 2000 mg L^{-1} of GA_3 for 24 h [61].

In agreement with the above-reported results, acid scarification followed by addition of GA_3 solution to the germination substrate and one week chilling was reported as a simple, efficient and cost-effective method for ensuring satisfactory seed germination in an experiment carried out in Kuwait [62], where a significant variation in germination percentage due to germination substrate was also evidenced.

The effects of different growing media and sowing depths on seed germination of caper have been more recently compared on *Capparis ovata* by Olmez and Olmez [63], who reported the highest germination percentage (51.1%) with peat+perlite+manure (2:1:1) at 1.0 cm sowing depth. Accelerated caper seed (cv. Común) ageing for 24, 48, 72 or 96 h at 45 °C was found to improve germination percentage (>90%) compared to non-aged seeds [64]. In research carried out in Syria on the effects of gamma irradiation on the germination of caper seed cultured in vitro [65], a significant effect of irradiation at the 100 Gy dose on dormancy breaking and germination (50%) of caper seeds was observed. In contrast, unsatisfactory germination results were observed [60] using gamma rays at different doses (10 to 50 KR) along with concentrated H_2SO_4 treatments for different durations. A synergistic effect of GA_3 (250 ppm) and KNO_3 (8000 mg L^{-1} for 24 h), useful to improve seed germination of caper (up to 72%), was observed in an Iranian experiment with seeds placed on filter paper [66]. Satisfactory caper germination percentage and germination rate (up to 75% and 1.35, respectively) were reported in another Iranian experiment, after treatment with sulfuric acid for 15 min, and 2000 ppm GA_3, under alternate 20–30 °C temperatures [67]. The use of salicylic acid and GA significantly increased germination and seedling growth in *C. spinosa* under drought stress [68].

The influence of the genetic factor on germination performance was studied, comparing seeds of *C. spinosa* L., subsp. rupestris with seeds of the subspecies spinosa, where the seeds of the former subspecies germinated earlier and to a greater extent than those of the latter subspecies [46]. In a parallel experiment, the effectiveness of the germination procedures was conditioned by the time elapsed between harvesting the seeds and their seeding. In fact, seeds' germination performance decreased with increasing storage period,

so that the highest germination percentages (>90%) were obtained within 30 days from seed collection [46].

The exposure of caper seeds to magnetic fields (125–250 millitesla) had a positive effect on seed germination (82%) when combined with the addition of GA to the substrate [69].

Lastly, Foschi and co-workers [70], comparing the germination performance of intact seeds, scrapped seeds, cracked seeds and broken seeds of different provenance, clearly demonstrated that caper seeds do not have a water-impermeable coat sensu stricto. In other words, caper seeds do not present a real physical dormancy but rather showed the presence of a physiological dormancy. In fact, with the use of 500 mg L^{-1} GA, they obtained that all viable seeds germinated. These observations led the authors (i) to suggest a sort of "push power" of the embryo, evoked by GA, able to reduce seed mechanical resistance to embryo expansion, thus allowing germination, and (ii) to conclude that imbibition of caper seeds is not a determining factor in their germination.

3. Vegetative Propagation

Vegetative propagation (syn.: multiplication, asexual or clonal propagation) is based on the capacity of shoots, leaves and roots, once detached from the mother plant, to regenerate the lacking part of the plant by emitting new roots or adventitious buds, or to join together (grafting) to form a new functional plant identical to the mother plant [71]. Therefore, compared to seed propagation, vegetative propagation of mature, selected plant material offers the main advantages, inter alia, to overcome both seedling juvenility and the undesirable heterogeneity that is found in plantations obtained by seed. Multiplication comprises self-rooting, layering, grafting and budding techniques. Among these, self-rooting is the most utilized for caper, although *C. spinosa* L. is commonly considered a difficult-to-root woody species, strongly dependent for the achievement of good results on plant material and seasonal and environmental parameters [72].

Different self-rooting techniques have long been tested and applied to *C. spinosa* and are currently adopted in Spain and in the Aeolian island of Salina. In these areas, despite the erratic response of rooting generally reported [18,37], caper is often propagated by stem cuttings. This is the standard method for 'Mallorquina' and 'Italiana' in Spain, and 'Nocella' and 'Spinoso' in Salina [16,26], even if plants obtained by cuttings are reputed by the farmers to be more susceptible to drought, at least in the first years of plantation [16]. Regarding the different types of cuttings, the worst results have been obtained in Spain and in Italy (Figure 4) with semi-hardwood cuttings collected and planted during August and September. Conversely, appreciable results have been obtained with hardwood and softwood cutting utilization. Woody cuttings, 12 to 30 cm long and 1.0 to 2.5 cm in diameter, are taken in autumn at the base of the mother plant, from 1-year-old wood. They are then stored in cold and humid conditions (sand stratification or cold room at 3–4 °C), after a fungicide treatment, until their planting (February) in the nursery section, covered by shading nets, with or without the application of indolebutyric acid (IBA) or naphthaleneacetic acid (NAA). Sometimes, two to three short longitudinal incisions are made at the base of the cuttings, and the use of bottom-heated systems is strongly recommended. Unfavorable results have been obtained with the 'Del País' variety, which may be considered recalcitrant. With the use of bottom-heated systems, 95% to 100% rooting for hardwood cuttings of 'Mallorquina' has been reported [37]. Softwood cuttings, 6 to 9 cm long, and >3 mm in diameter, are taken in spring (April) and are exclusively rooted in the nursery where the use of bottom-heated systems and intermittent mist propagation techniques are recommended [37].

Figure 4. Vegetative propagation by rooting during experiments at the University of Palermo, Italy.

Variation in the rooting potential of caper cuttings has been generally reported as affected by the type of cutting, cutting harvest time and by the PGR and substrate utilized [73,74]. In Italy, dipping the basal end of the cutting into 1500–3000 mg L^{-1} IBA solution enhanced rooting, depending on the type of cutting and the season in which cuttings were taken, with rooting percentages under mist propagation or heated bed systems between 55% and 75% with hardwood cuttings taken in March–April, corresponding to the initial bud–break phase [75,76]. On the other hand, herbaceous (softwood) cuttings, taken at the beginning of the vegetative cycle (April), showed more satisfactory results, with 90% rooting success, in the case of spineless caper biotypes rather than with spiny ones [77]. Cutting diameter has been generally reported to be determinant for the result in various studies [16,37,78] and, ordinarily, the best results have been achieved with values at least 5–6 mm or even higher, and not below 3 mm, for hardwood and softwood cutting, respectively. A positive correlation between cutting diameter and rhizogenesis success has been reported for hardwood and semi-hardwood cuttings, as well as a positive effect of sealing the top- and the bottom-end of cuttings with paraffin [46].

Besides depending on the type of cuttings, rhizogenesis results have varied depending on the presence of leaves (leafy or leafless cuttings). Leafy cuttings treated with 6000 and 9000 mg L^{-1} IBA showed higher rhizogenesis percentages (67%) than leafless ones (61%) [29]. Additionally, in the same research, IBA positively affected root number per cutting more than NAA, but both PGRs did not differ in terms of cuttings' root length, which was significantly higher than in the controls.

The use of irradiation with low doses of gamma radiation (10 Gy) stimulated (in vitro) rooting of shoots from 75% to 100% [65].

Grafting, as a propagation technique, is the least used method in capers. In Spain, satisfactory results (60% scion take) were obtained using bark grafting in mature plantings. Nurseries generally whip-graft with survival rates of 73% [37]. Both systems are now considered excessively expensive and are, therefore, no longer practiced.

4. In Vitro Propagation

The advancement of scientific knowledge and the development of practical techniques have permitted, in recent decades, the constant progress of in vitro plant biotechnologies, particularly those useful to multiply hard-to-propagate woody species by conventional methods [79]. In vitro growth and development are promoted by PGRs which are responsible for cell division and growth. PGRs (auxin and cytokinin) regulate the development of different plant organs during in vitro growth. The auxins (IAA, IBA, NAA or 2,4-dichlorophenoxyacetic acid (2,4-D)) induce cell elongation and tissue swelling, cell division (callus proliferation) and the formation of adventitious roots; the cytokinins (kinetin, ben-

zylaminopurine (BAP), meta-topolin (mT), 2-isopentenyladenine (2iP) or zeatin (ZEA)) stimulate growth and development. They also improve cellular division and induce the formation of adventitious shoots by decreasing the apical dominance.

Even in the case of *C. spinosa* L., micropropagation has recently offered an interesting and promising alternative for rapidly obtaining, starting from a single explant, highly uniform, pathogen-free plant material, suitable both for specialized plantings and ex situ conservation (Figure 5).

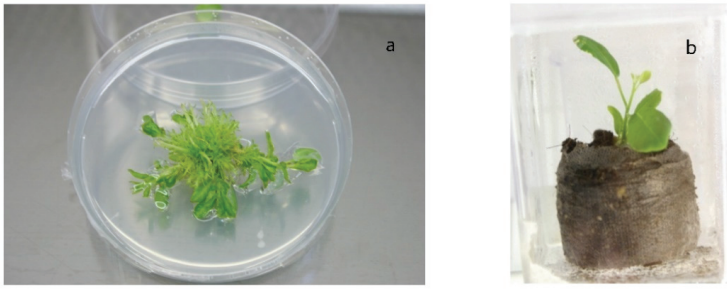

Figure 5. In vitro propagation carried out at the University of Palermo. Experiments on proliferation (**a**) and on the initial phase of ex vitro acclimatization (**b**).

In vitro *C. spinosa* L. propagation was reported for the first time in 1984 [80] and soon after, it was followed by other studies which mainly dealt with nodal segments as starting material [56,65,80–83]. Others have reported successful micropropagation protocols regarding other caper-related diverse species, beyond the scope of the present review, such as, for example, *C. decidua* (Forsk.) Edgew [84,85], or *C. orientalis* Duh. and *C. leucophylla* DC. [86,87].

Limiting our analysis to *C. spinosa* L., it was evident that in most of these studies the best results, in terms of the induction of axillary growth, were obtained with cytokinins (mainly BAP) and auxins, used either alone or in combination. Due to *C. spinosa*'s reputation as a hard-to-root species, the in vitro rooting phase is considered critical for caper propagation. High rooting responses (70%) of local caper populations of unreported origin were obtained in 1990 [80] using IAA at 5.25 mg L^{-1}, even though there were marked differences among different tested auxins and concentrations in terms of rooting efficacy. Subsequently, other authors have reported high success rates (80–100%) for the rooting phase. In Lebanon, the rooting percentage of a local ecotype was improved up to 80.5% with IBA at 5 mg L^{-1} for 10 min [81] and in another experiment on explants from a Lebanese-selected mature shrub, a high rooting response (87–92%) of shoots was obtained after a 4 h pulse treatment with IAA at 100 mg L^{-1} [88]. In Tunisia, on explants coming from one-year-old mother plants of a local ecotype, the maximum rooting (80%) was obtained with IAA at 1.5 mg L^{-1} [77]. On plant material proceeding from Sicily, rooting percentages up to 87% with IBA at 100–200 mg L^{-1} were reported [89]. In Jordan, the best auxin for in vitro rooting (80%) of wild caper plants material was IAA at the level of 5.0 mg L^{-1} [82]. In Syria, the effect of gamma irradiation on the growth of wild caper explants was studied. A 10 Gy dose of gamma irradiation stimulated in vitro growth of shoots up to 200% [65].

In a study on plant material proceeding from Jordan, maximum root formation percentage (60%) was obtained with 2.0 mg L^{-1} IAA, but similar successful results with IBA and NAA at various concentrations were also reported [90]. On plant material obtained from a mature plant of a Sicilian selected genotype (code ICAORL2), grown in the collection of the University of Palermo, a high percentage (93.7%) of well-rooted plantlets was achieved with the synthetic phenylurea N,N′-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) (1 μM) [78]. In a study carried out in Saudi Arabia, aimed at exploring in vitro culture protocols for ex situ conservation purposes, using axillary buds of wild *C. spinosa* L.

plants, the highest percentage of rooted shoots (56.7%) was observed on half-strength MS medium without NAA and on full MS with 1.5 mg L^{-1} NAA [91].

A new technique to regenerate caper plants starting from floral explants (unfertilized ovules) on MS medium, supplemented with 88 mM sucrose and 13 μM BAP, was successfully developed by Carra and co-workers [92]. Multiple shoots were obtained on MS medium supplemented with BAP and IBA. The best rooting results (100% of rooted explants) were obtained when explants were dipped for 10 min in 50 μM IBA solution and successively maintained in PGR-free medium. In another experiment carried out on selected Sicilian genotypes, IBA showed better rooting performances than IAA and NAA at all the tested concentrations (1, 5, 10 μM). The best rooting rate (93.4%) was achieved with IBA at 5 μM [93].

The usefulness of PlantForm bioreactors as an alternative to traditional solid-substrate techniques was tested on plant material obtained from mature plants of three Sicilian selected accessions, named 'Sal 39', 'Sal 37' and 'Sal 35', grown on the Aeolian island of Salina [94]. The results show that the caper shoots from bioreactors demonstrated good adaptability and better growth rates. Furthermore, the relative growth and real proliferation rate of the caper explants were higher when using mT (eight new proliferated shoots after 60 days) than when using BAP as a PGR (five new proliferated shoots after 60 days).

In a successive experiment, carried out on plant material from the three same aforementioned accessions, low rooting efficiency with IBA and no response to rooting with IAA was found at the tested concentrations. The maximum rooting performances were observed for only one of the tested accessions ('Sal 39') when explants, proceeding from liquid culture in PlantForm, were supplemented with NAA at 0.75 mg L^{-1} + IBA at 0.25 mg L^{-1}, indicating the importance of the role exerted by the cultural conditions during the proliferation phase. Additionally, these results confirm that mixtures of root-promoting substances are sometimes more effective than either component alone.

Finally, it must be observed that rooting induction often required high levels of auxins which, on the other hand, may stimulate callus formation [88,93]. In these conditions, the abundant presence of callus delays root formation [90] and is unfavorable for ex vitro transfer. Moreover, high concentrations of auxins could be inhibitory for root growth [94] and may be the cause of somaclonal variation in the production cycle [95].

Altogether these results clearly confirm, as has already been pointed out [85], that for an individual genotype, or even the same local population, the rate of rooting is strongly determined by the type and concentration of auxin, but, considered as a whole, they also seem to indicate that no one protocol, although optimal with a specific accession, may fit all genotypes [94,96].

5. Conclusions

The caper (*C. spinosa* L.) has long been considered a difficult-to-propagate species. In this review, a comparative analysis carried out on the most significant scientific contributions to the issue of both conventional and innovative propagation has led us to prudently confirm this reputation only in part. In fact, far from being able to consider all the specific problems related to the different propagation technologies to be overcome, it has nevertheless clearly emerged that, given the right conditions and the appropriate protocols applied to specific accessions, more than satisfactory results are possible with most of the conventional and innovative available technologies. Furthermore, it is probable that erratic or unsatisfactory propagation results, variously reported, must be linked to the extreme heterogeneity of the plant materials used, rather than to intrinsic limits of the adopted technologies. In this context of the high variability existing within the taxon, we can still consider *C. spinosa* L. as a difficult-to-propagate species, sensu lato.

However, from an agronomic point of view, the unreliability and low degree of transferability of propagation research results are strongly indicative that the caper industry, including related nursery systems, is still in its infancy. This is also supported by the evidence of the extreme, unjustified emphasis that has been reserved so far for sexual

reproduction, as emerged from the present review. In woody perennial fruit plants, seed propagation is usually reserved only for rootstocks and genetic improvement, whereas vegetative (clonal) propagation is the most frequent and appropriate method for specialized woody crops to assure specific phenotypes of agronomic and commercial interest. Caper, as a crop, has a recent history and can be considered only a semi-domesticated or a pre-domesticated crop, due to the substantial absence of breeding and "crop improvement" within the cultivated accessions, i.e., the absence of real "cultivars". Even if the caper is the subject of intense trade between producing countries and importing countries (mainly the USA and the UK) it can also be, therefore, included among the "orphan crops", since it has certainly received, so far, less attention in terms of research, mainly with respect to the selection of well-adapted, valuable and easy-to-propagate candidate cultivars. For the improvement and modernization of the caper culture it is, therefore, essential that research concentrates efforts on genetic improvement programs, starting from the valorization of the vast genetic potential existing within the germplasm, both in nature and under cultivation.

The ideotype should include high productivity of a large number of deep green, rounded, easy-to-pick flower buds; tender shoots needing heavy pruning with rapid regrowth; soft, light green, oval-shaped caper berries with few seeds; short internodes; good resistance to pests and diseases; spineless or with a scarce number of spines; and, ultimately, easy vegetative propagation.

Unfortunately, the drastic decline suffered by the caper industry—which decreased in Spain, for instance, from over 7500 hectares in 1984 to 550 in 2008 and to 483 hectares in 2018—suggests an equally drastic drop in interest in research investments for caper in the near future. On the other hand, imports from North Africa, and other countries where labor is cheaper, but where caper is mainly harvested in the wild, will most likely increase the risk of genetic erosion to unsustainable levels. To try to counter this trend, the improvement of current conventional and innovative propagation techniques can play an essential role both in the conservation of genetic resources and in varietal crop cross-breeding. At the same time, decisive is the inversion of the tendency to cultivation abandonment, potentially driven by a renewed agronomic research interest.

However, on the basis of the state of the art discussed in the present review, it is conclusively possible to assert that the caper, as a crop, must no longer be reputed as a difficult-to-propagate crop sensu stricto, given the most modern and appropriate technologies available and, above all, once a range of proven reliable cultivars are made available.

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Seed Micromorphology, *In Vitro* Germination, and Early-Stage Seedling Morphological Traits of *Cattleya purpurata* (Lindl. & Paxton) Van den Berg

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Abstract: In the context of a symbiotic plant-fungus interaction study concerning *Cattleya purpurata*, we focused on some aspects of seed morphology and biology, and the early stages of seedling development. Seed morphology was characterized using light and scanning electron microscopy. In vitro seed germination capability was evaluated, comparing symbiotic and asymbiotic methods. The morphology of the seeds was overall comparable to that of other congeneric species, showing classical adaptations related to the aerodynamic properties and to the wettability of seeds, but calcium oxalate druses were identified inside the suspensor cells. Asymbiotic seed germination was successful in all tested media (17.1–46.5%) but was higher on 1/2 Murashige & Skoog. During symbiotic interaction with the fungal strain MUT4178 (*Tulasnella calospora*), germination rate was significantly lower than that obtained with the best three asymbiotic media, suggesting a low fungal compatibility. Seedling morphology was in line with other taxa from the same genus, showing typical characteristics of epiphytic species. Our observations, in particular, highlighted the presence of stomata with C-shaped guard cells in the leaves, rarely found in Cattleyas (where usually they are reniform), and confirm the presence of tilosomes in the roots. Idioblasts containing raphides were observed in both roots and leaves.

Keywords: crystals; microscopy; mycorrhizal fungi; orchids; suspensor

1. Introduction

Orchidaceae, with about 28,000 species and new ones being discovered every year, is one of the largest families of flowering plants [1]. The great majority of species is native to tropical or sub-tropical areas, but the family can be considered cosmopolitan, being spread over the five continents, with a distribution range that extends from the Arctic Circle to sub-Antarctic islands [2]. Orchids have interesting and often strict interactions with specialized pollinators and mycorrhizal fungi [3–5], and are at risk of extinction by intrinsic and extrinsic factors linked with habitat degradation [6], including weed invasion, grazing, altered hydrology, and altered fire regimes.

Among the Epidendroideae subfamily, the subtribe Laeliinae is strictly neotropical and comprises about 50 genera with about 1500 species [7,8]; it is one of the largest subtribes in the family after Pleurothallidinae and Oncidiinae [9]. Due to its morphological variability and taxonomic divergence [9], Laeliinae has been the subject of great taxonomic reviews

over the last few decades; in this subtribe, Dressler [7] included the genera *Cattleya* Lindl., *Laelia* Lindl., *Schomburgkia* Lindl., and *Sophronitis* Lindl. Van den Berg et al. [9,10], thanks to a phylogenetic analysis on ITS regions and considering the hybridization capability within Laeliinae, supported the inclusion of *Schomburgkia* and *Sophronitis* species in *Laelia* and *Cattleya*, respectively. For the above-mentioned reasons, Brazilian *Laelia* species, previously referred to as *Hadrolaelia* [11], have also been included in the genus *Cattleya* [12].

Cattleya purpurata (Lindl. & Paxton) Van den Berg (syn. *Laelia purpurata*) is a Brazilian orchid species that grows widely in the country and has been ranked as endangered (EN) by IUCN (International Union for Conservation of Nature) in the State of Rio Grande do Sul [13], due to its over-collection for commercial purpose [14]. The species has been the focus of many studies due to its high economical value; it is considered one of the flag flowers of Brazil and nowadays it is in collections all over the World, with several cultivars and varieties [15]. As recently reported by Caballero-Villalobos et al. [16], the species is nectarless and self-compatible, although it is pollinator-dependent. Some morphological investigations on the vegetative portions of this species have been carried out by Silva Júnior et al. [17] and Gallo et al. [18]. The former studied leaf characteristics and chlorophyll content of this species after the applications of different urea concentrations during *in vitro* experiments. Gallo et al. [18] performed an anatomical investigation on seed and protocorm from six species belonging to the genus *Cattleya*, *C. purpurata* included. Anatomical investigations provide important information to assess species identity, to better understand physiological processes, or to study phylogenetic relationships between taxa [19]. This information, which can be achieved considering both the vegetative and reproductive portions of a species, could also be used to improve knowledge and be applied in micropropagation techniques and conservation programs [18].

New propagation techniques have been proposed over the last decades to optimize asymbiotic and symbiotic seed germination, and to promote seedling acclimatization [15,20,21]. However, contrary to what is known about terrestrial species [22–24], few details are available concerning mycorrhizal associations in tropical orchids, especially in Laeliinae occurring in different environments (i.e., [25,26]).

In this work, which has been carried out in the framework of a plant-fungus interaction study [27], our aim was to obtain more morphological information regarding *C. purpurata* seed traits and test seed germination via both asymbiotic and symbiotic techniques, in the latter case using a fungal strain of the well-known orchid mycorrhizal (OM) fungus *Tulasnella calospora.* Seedlings grown on different media and growth conditions were then characterized from an anatomical and micro-morphological point of view in order to collect further data concerning this stage of development and to improve propagation techniques to be used in scientific and conservation programs.

2. Materials and Methods

2.1. Flower Hand Pollination and Seed Collection

Capsules were obtained by hand pollinated plants of *Cattleya purpurata* cultivated by the Azienda Agricola Nardotto e Capello in Camporosso (Imperia, Italy), 43°48′18″00 N, 07°37′43″32 E. At the time of capsule maturation, indicated by a yellowish color, three capsules were collected, and seeds were stored at 4 °C in a paper envelop until use [28].

2.2. Seed Sowing

After a sterilization in 1% NaClO solution, seeds were rinsed three times in sterile dH_2O for 5 min and then sowed on six different asymbiotic seed germination media: 1/2 strength of Murashige and Skoog [29], including vitamins (Duchefa) enriched with 50 mL/L of coconut water; Knudson C [30] orchid medium (Duchefa); Malmgren Modified medium [31]; M551 (Phytotechnology) enriched with 1mg/L BAP, which are commonly used for the propagation of epiphytic and terrestrial orchids [32]; and CG (CG0) and CG enriched with 50 mL/L (CG50) and 100 mL/L (CG100) almond milk, a new medium that showed to improve *in vitro* seedling development in both epiphytic and terrestrial

orchids [32]. All the media were supplemented with 2 g/L of activated charcoal and a total concentration of 10 g/L of sucrose; pH was adjusted to 5.8. Seeds were also sowed on an oat agar medium (OA), as described by Ercole et al. [33], with a mycorrhizal fungal strain of *Tulasnella calospora*, isolated at the University of Turin from *Serapias vomeracea* [34] and deposited in the mycological collection of the University of Turin (Mycotheca Universitatis Taurinensis, accession number MUT4178). Eight replicates for each medium were periodically checked under light microscope to assess germination percentage. The final germination percentage, recorded 70 days after seed sowing, was evaluated as the overall mean of single observations ± SE. Seedlings were then moved to jars with the same media for three months before recording morphological data.

2.3. Microscopy

2.3.1. Plant Material

Nine months after sowing (after transferring seedlings on fresh media every three months), fully developed roots and leaves from each medium were sampled. Roots and leaves were cut about 1 cm from the apex and about half of their length, respectively. Plant material was prepared to perform micro-morphological investigations according to the following methods.

2.3.2. Light Microscopy

Light microscopy (LM) analysis was carried out on seeds, seedling leaves, and roots. Seeds were directly mounted onto a microscope slide after being rinsed in 5% NaClO solution for 1 h, and were then observed with a Leica DM 2000 transmission-light microscope coupled with a computer-driven DFC 320 camera (Leica Microsystems). Seed and embryo length, length/width (L/W) ratio, number of cells along the entire seed longitudinal axis, and length and number of cells of the suspensor were recorded for 100 seeds. Seed descriptive terminology has been used according to Barthlott et al. [35].

Transversal hand-sections of fresh leaves and roots obtained by a razor blade were stained with both TBO (Toluidine Blue O), to discriminate cell wall structures, and acid Phloroglucinol, to highlight xylem and lignified structures in general. The seeds and transversal sections of leaf and roots were also examined under a polarizing filter. In addition, to localize suberin and lignin, transversal sections of roots were stained with Fluorol Yellow 088 and observed under UV light.

2.3.3. Scanning Electron Microscopy

Seeds were incubated overnight in 100% ethanol, placed on aluminum stubs covered with double-sided carbon tape, and dried at room temperature, as performed by Calevo et al. [36]. Stubs were then sputter-coated with 10 nm gold particles and directly observed with a Vega3 Tescan LMU Scanning Electron Microscope (SEM) (Tescan USA Inc., Cranberry Twp, PA, USA) at an accelerating voltage of 20 kV, coupled to an X-ray Energy Dispersive System (EDS) Apollo XSD (Tescan USA Inc., Warrendale, PA, USA). A detailed assessment of seed ornamentation was carried out, considering the ornamentation of the periclinal walls and the type of anticlinal walls [37].

For SEM analysis of leaves and roots from in vitro grown seedlings, transversal sections of fresh material were fixed in FineFIX working solution (Milestone s.r.l., Bergamo, Italy) with 70% ethanol, and left overnight at 4 °C [38]. Sections were then dehydrated for 1 h through graded ethanol series from 70% to 100% at 60 min intervals, and finally dried using a Critical Point Dryer Processor (K850CPD 2M Strumenti S.r.l., Roma, Italy). Specimens were then mounted on stubs, as previously performed on seeds, to carry out micromorphological analyses.

2.4. Data Analysis

Germination data were statistically analyzed by ANOVA followed by Fisher's probable least-squares difference test, with a cut-off significance at $p \leq 0.001$.

Embryo and seed volumes were calculated following the equations reported in [37]:

$$\text{Embryo volume (EV)} = 4/3\ \pi ab^2 \quad (1)$$

$$\text{Seed volume (SV)} = 2(\pi/3\ r^2h) \quad (2)$$

where a = 0.5 × embryo length (or major axis); b = 0.5 × embryo width (or minor axis); r = 0.5 × seed width (or minor axis); and h = 0.5 × seed length (or major axis).

The morphological data of seeds were analyzed using the R environment [39]. Correlations between seed and embryo volumes were analyzed by means of the cor.test function, using the Spearman's rank correlation coefficient rho [40,41]. Measures were expressed in μm ± SD.

3. Results

3.1. Seed Germination

Cattleya purpurata seeds germinated on all the tested media. The best medium for asymbiotic germination was 1/2 MS, with a germination percentage of 46.5 ± 6.4%. All the other asymbiotic media yielded lower germination percentages, ranging from 17.1 ± 2.0% (CG0) to 26.3 ± 4.3% (KC). The use of MUT4178 fungal strain of *T. calospora* resulted in a germination of 18.3 ± 4.0%, statistically comparable with all the variants of CG medium (Table 1).

Table 1. Germination mean percentages (± Standard Error) (n = 8) recorded 70 days after seed sowing of *Cattleya purpurata*. Data were analyzed by ANOVA followed by Fisher's probable least-squares difference test with cut-off significance at $p \leq 0.001$. Percentages with the same letters are not significantly different from each other.

Medium	Germination %
1/2 MS	46.5 ± 6.4 a
KC	26.3 ± 4.3 b
M551	24.1 ± 2.6 b
CG0	17.1 ± 2.0 c
CG50	21.4 ± 2.9 bc
CG100	22.9 ± 5.3 bc
OA + MUT4178	18.3 ± 4.0 c

3.2. Morphology

3.2.1. Seed Morphology

Cattleya purpurata seeds, beige in color, are elongated (Figure 1a) and present a L/W ratio of 5.33 ± 0.62. They showed an average seed length (excluding the suspensor) of 800.47 ± 97.42 μm, an average width of 154.75 ± 22.37 μm, an embryo length of 331.59 ± 48.04 μm, and an embryo width of 127.22 ± 19.98 μm (Table 2). The comparison of seed and embryo volumes showed a significant positive relationship (R = 0.74, $p < 0.001$) (Supplementary Figure S1). The average suspensor length was 302.52 ± 64.53 μm; no significant relationship was found between suspensor length and both seed and embryo length and either between suspensor length and seed and embryo volumes.

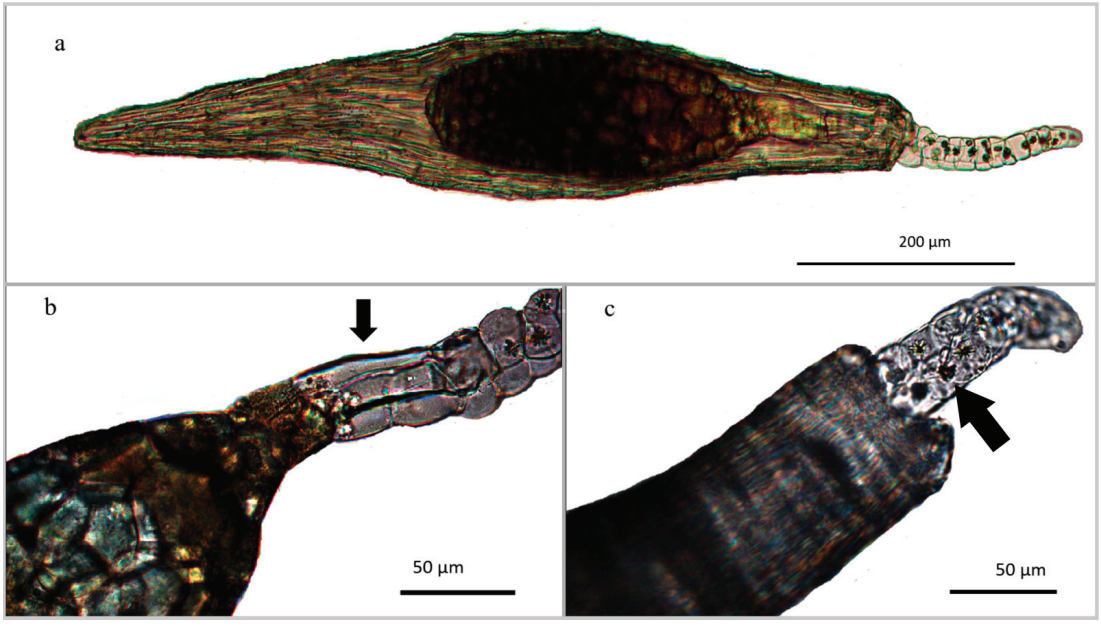

Figure 1. Light microscopy images of *Cattleya purpurata* seed. (**a**) LM overview of NaClO-treated seed, in which embryo is well distinguishable within the seed coat; (**b**) detail of the embryo body-suspensor connection (arrow) composed of 2–3 elongated cells. (**c**) untreated seed: View of the insertion of the suspensor inside the micropyle. Spherical druses are present in each cell of the suspensor (arrow).

Table 2. Means of seed morphological parameters (n = 100) and seed features of *Cattleya purpurata* (± Standard Deviation).

Seed Type	Elongated
Seed length (µm)	800.47 ± 97.42
Seed width (µm)	154.74 ± 22.37
Seed L/W ratio	5.33 ± 0.62
Embryo length (µm)	331.59 ± 48.04
Embryo width (µm)	127.22 ± 19.97
Suspensor length (µm)	302.51 ± 64.53
Seed testa cells	Without intercellular spaces
N° cells in longitudinal axis	>5
Suspensor layer	2–3 cell thick
Crystals	Calcium oxalate druses in the suspensor

Clarifying treatment with NaOCl allowed us to verify that seed coat was monolayered and that there were no intercellular gaps. Concerning *testa*, more than five cells were present along the seed longitudinal axis. These cells were elongated; their periclinal cell walls presented verrucosities and granular epicuticular coverings, while their anticlinal walls were occasionally elevated. As shown in Figure 2a,b, the seed coat enveloped the embryo, which showed a relatively long suspensor. Two-three elongated cells connected the embryo body with the portion of suspensor extending beyond the micropyle (Figure 1b, arrow).

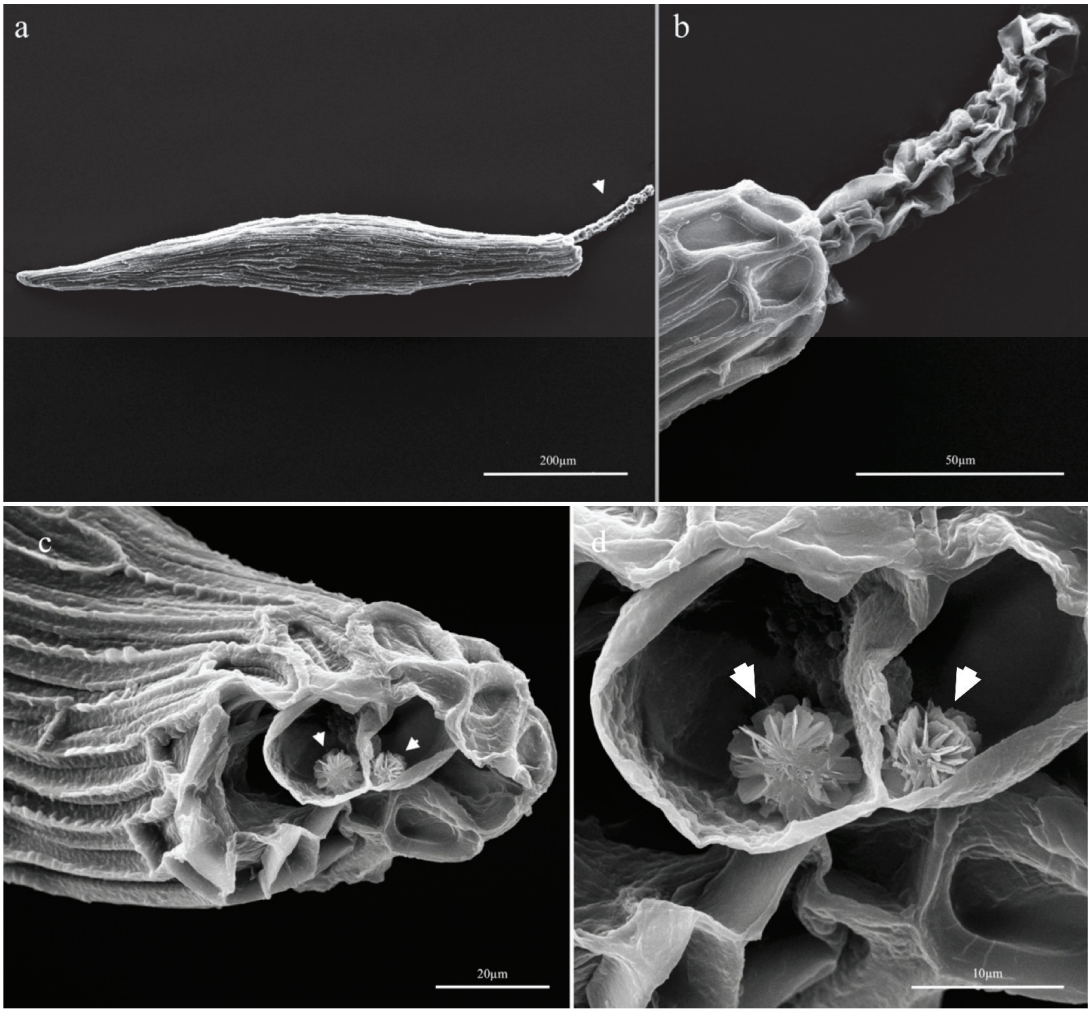

Figure 2. SEM micrograph of *Cattleya purpurata* seed. (**a**) overview of the seed showing the suspensor (arrow); (**b**) detailed view of the suspensor made of a 2–3 cells-thick layer; (**c**) cutaway of the suspensor highlighting the presence of druses (arrows) inside each cell. At higher magnification, the verrucose microrelief and the granular ornamentation of the seed coat cells are also visible; (**d**) particular of the druses (arrows).

The presence of spherical druses (6–8 nm in diameter) within the seed suspensor cells was recorded by both light (Figure 1a,c) and scanning electron microscopy (Figure 2c,d). SEM-EDS analysis of these crystals showed a high calcium peak, typical of the calcium oxalate spectrum (Supplementary Figure S2).

3.2.2. Root Sections

Microscopical analyses highlighted that the structure of roots sampled from *C. purpurata* seedlings grown on different substrates was qualitatively similar, except for the increased presence of hairs in individuals from CG50 and CG100 media (not shown). Velamen was two to four cells wide (Figure 3a); these cells appeared large, polygonal shaped, and radially elongated, and showed helical-banded wall thickenings (Figure 3b,g).

Exodermal cells were moderately large, polygonal-shaped, and ∩-thickened (Figure 3b,d). Short thin-walled passage cells were scattered in the exoderm, near tilosomes of tufted type (Figure 3b, arrows) (according to Pridgeon et al. [42]). In the parenchymatous cortex, the presence of chloroplasts (Figure 3d) and raphides (Figure 3e) was recorded. Cortical cells varied in form and size: elements near to the endodermis were smaller than those of the middle layers (Figure 3a,d); some parenchymatous cells appeared more or less circular, while others were polygonal-shaped (Figure 3a,b,d). As confirmed by means of polarized light (not shown) and by Fluorol Yellow staining, some elements of the cortex presented suberized thickenings in cell walls (Figure 3c, arrows). Endodermis cells were square to tetragonal-shaped; passage cells were visible in correspondence of phloem clusters, while the other endodermis cells appeared O-thickened (Figure 3c). The pericycle presented thin-walled polygonal-shaped cells alternating with protoxylematic cell elements. This layer surrounded the central vascular cylinder characterized by a polyarch actinostele, which showed 10 xylem arches. In the root's central region, a parenchymal tissue composed by circular cells alternating with lignified elements was present (Figure 3c).

Roots of seedlings obtained from symbiotic cultures showed the presence of mycorrhizal hyphae on the external surface (Figure 3f). The beginning of hyphal coiling was also well visible on the wall thickening of the velamen cells (Figure 3g). Root features are summarized in Table 3.

Table 3. General seedling's root and leaf anatomical characters of *Cattleya purpurata* observed on all tested media (unless otherwise indicated).

Root	**Characters**
Hairs	From few to numerous if roots belonged to plants grown in CG50 and CG 100
Velamen cells	Moderately large, polygonal shaped, arranged in 2–3 layers. Helical thickenings in cell walls
Exodermis cells	Large, polygonal-shaped, arranged in monolayer. ∩-thickened.
Cortex cells	Circular to polygonal shaped. Variable size. Presence of chloroplasts. Some elements show thickenings in the cell walls.
Central cylinder	Endodermis cell walls square to polygonal shaped, O-thickened; passage cells in correspondence of phloem; thin-walled pericycle cells alternating with protoxylematic elements.
Stele	Polyarch actynostele (10 arches)
Crystals	Raphides in the parenchymatic cortex cells
Other features	Tufted tilosomes near short thin-walled passage cells scattered in the exodermis
Leaf	
Cuticle	Granular ridged; 7–10 μm thick;
Stomata	Tetracytic apparatus. C-shaped guard cells. Present in the abaxial surface. Mean stomatal length: 23.5 ± 2.9; mean stomatal width: 26.5 ± 2.6.
Epidermis	Cells rectangular/hexagonal shaped. Generally, one-layered (two-layered in the midvein zone)
Hypodermis	Present in correspondence of the midvein
Mesophyll	Dorsiventral in correspondence of the midvein, homogeneous towards the margins. Seven vascular bundles for each side of the midvein, arranged in a row. Transverse veins connecting principal vascular bundles. Abaxial row of schlerenchymatic fibers. Banded mesophyll cells.
Crystals	Idioblasts containing raphides

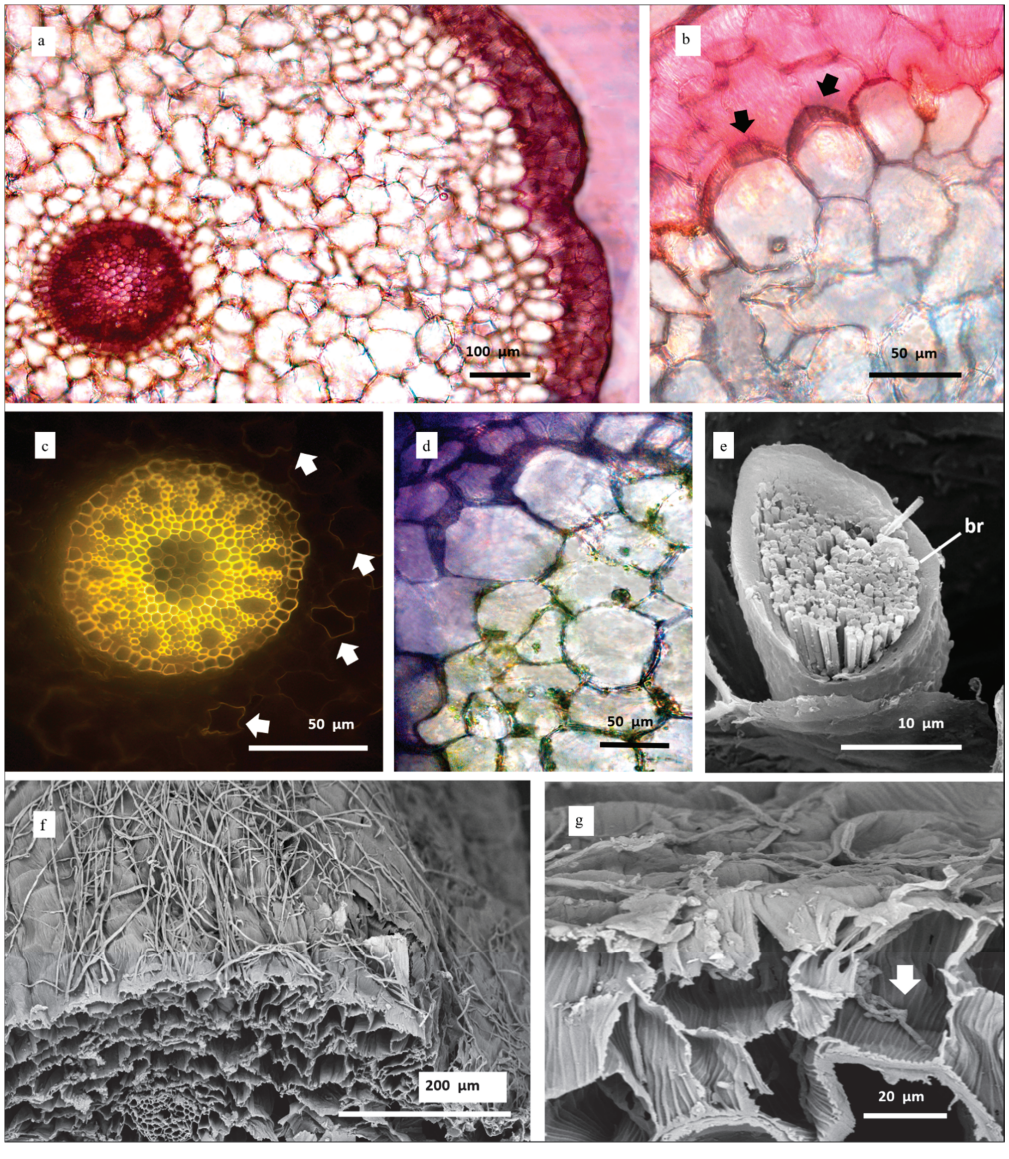

Figure 3. Root anatomy of *Cattleya purpurata* seedlings. Representative seedlings obtained on 1/2MS were selected for panels (**a**–**e**) and from OA medium for panels (**f**,**g**). (**a**) overview of the root's transversal section stained with Phloroglucinol-HCl. Velamen is composed by a 2–3 cells wide layer; exoderm cells appear large, polygonal, and ∩-thickened; cortex cells from middle layers are larger than those near to the exoderm and to the endoderm. The vascular cylinder is composed by a polyarch actynostele; (**b**) tufted tilosomes (arrows) observed in the endovelamen-exoderm region; (**c**) Fluorol Yellow staining: detail of the central vascular cylinder characterized by a polyarch actinostele. The central parenchymatic tissue is composed by circular cells alternating with lignified elements. Cortex elements showing suberized cell walls are also visible (arrows); (**d**) chloroplasts within cortex cells; (**e**) SEM photograph of bundledraphides (br) within a cell of cortical parenchyma; (**f**) SEM image of mycorrhizal hyphae in the first stages of colonization of a root obtained from symbiotic culture; (**g**) the beginning of hyphal coiling inside a velamen cell (arrow). Banded wall thickenings of velamen cells are also distinguishable.

3.2.3. Leaf Surface and Transversal Sections

The media used for seed germination and seedling development did not lead to structural changes in leaves. Leaf surfaces were composed of mostly rectangular to hexagonal cells and showed a ridged, granular cuticle, 7.0 ± 1.3 µm thick in the abaxial surface and 10.0 ± 1.6 µm in the adaxial one (Figure 4a,b). Tetracytic stomata with C-shaped guard cells were present only in the lower epidermis; the stomatal aperture was parallel to the longitudinal axis of the leaf (Figure 4a). Mean stomatal length was 23.5 ± 2.9 µm, while mean stomatal width was 26.5 ± 2.6 µm. In transversal section, on both sides of the midvein, seven vascular bundles arranged in a row were visible (Figure 4c). The central midvein (Figure 4c,d), positioned near the lower surface, was surrounded by several hypodermal cell layers (not shown). In correspondence of the central midvein, the epidermis was bistratified. The leaf presented a dorsiventral mesophyll at the level of the central midvein and a homogeneous one towards the margins. Parenchyma cells gradually increased in diameter while approaching the central portion, where isodiametric and elongated thick-walled cells were present near the midvein, resembling a palisade parenchyma (Figure 4c). In many cases, we observed vessel elements developed transversely, which connected the small veins (Figure 4e). Vascular collateral bundles showed sclerenchyma caps at both xylem and phloem poles, but predominantly at the phloem one (Figure 4d,e). Little sclerenchyma fiber bundles were present as an abaxial row (not shown), while two clearly distinguishable bundles were positioned at each leaf margin: a little bundle in the margin tip and a larger one more internal (Figure 4c,f). Idioblasts containing raphides were interspersed in the mesophyll (Figure 4f, arrow). Leaf features are summarized in Table 3.

As highlighted by SEM analyses, some mesophyll cell walls were banded (Figure 4g).

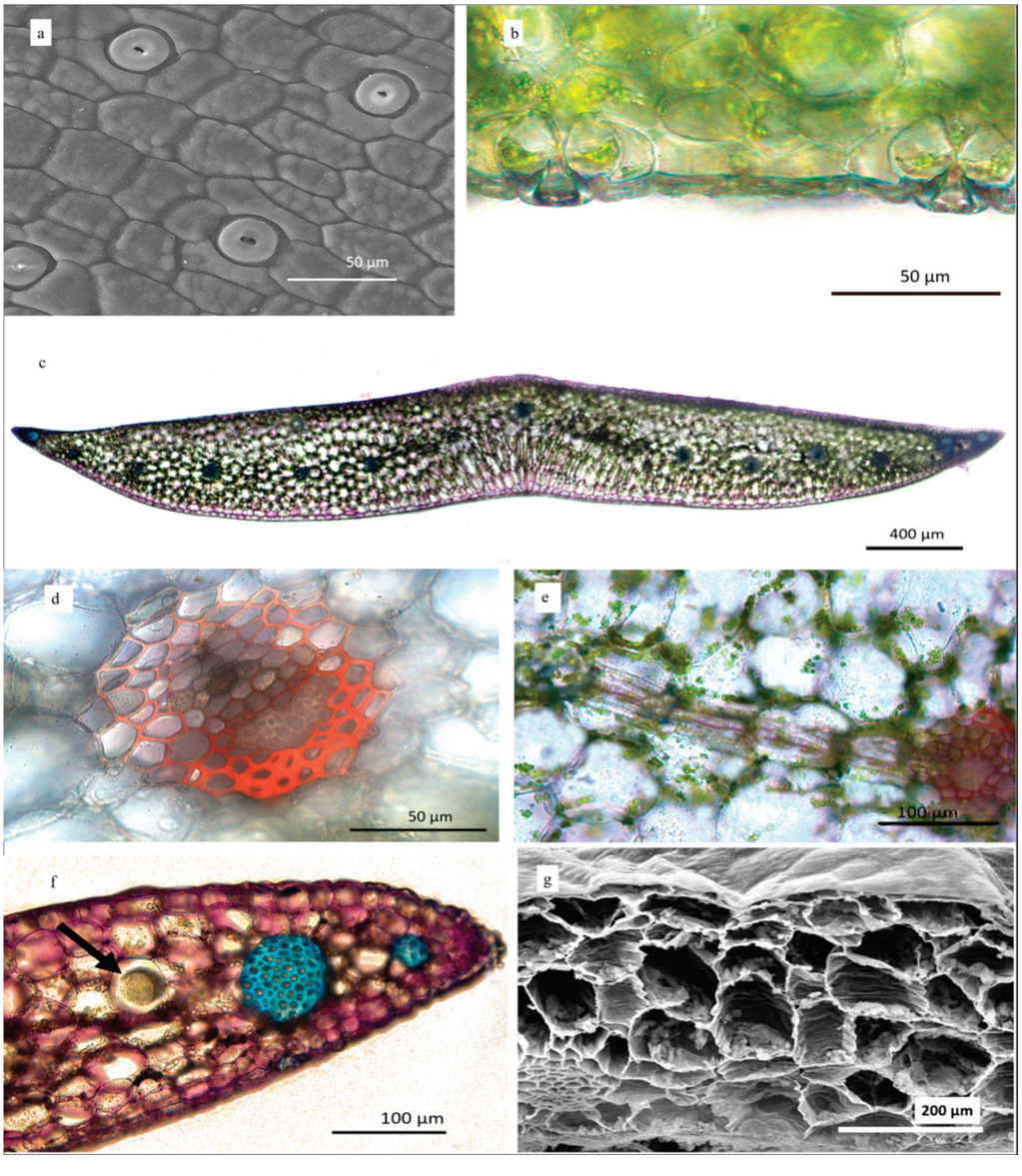

Figure 4. Leaf anatomy of *Cattleya purpurata* seedlings. Representative seedlings obtained on M551 medium were selected for images. (**a**) SEM picture of the lower surface of the leaf, revealing hexagonal cells covered by a thick granular cuticle and the tetracytic stomatal apparatus with C-shaped guard cells; (**b**) transversal section: detail of the leaf's abaxial portion, in which stomata are visible. Cuticle is 6–9 µm thick; (**c**) transversal section stained with TBO. Seven vascular bundles arranged in a row are visible. The central midvein is positioned near the lower surface. A dorsiventral mesophyll is present at the level of the central midvein, while a homogeneous one is distinguishable towards the margins. Two sclerenchymatic bundles are present at each leaf tip; (**d**) View of the central midvein stained with HCl-phloroglucinol; (**e**) transversely developed vessel elements, connecting with a vascular bundle red-stained with HCl-phloroglucinol; (**f**) transversal section of the leaf stained with TBO. The two sclerenchymatic bundles are visible near the leaf margin; (**g**) SEM picture of the mesophyll in cross section: banded thickenings are distinguishable in some cells.

4. Discussion

As observed by Gallo et al. [18] and confirmed by our analyses, the seed coat of the studied species consists of only one cell layer. As reminded by the latter authors, the reduced and membranous seed coat of Laeliinae seeds and the air space between the tegument and the embryo is an adaptation related to the aerodynamic properties and to the wettability of seeds [37,43]. While a significant correlation has been found between seed and embryo length, suspensor length was not correlated to seed or embryo size, indicating that suspensor dimension does not influence seed development in *C. purpurata*.

Although Gallo et al. [18] already described the long multicellular suspensor in *C. purpurata* and other specimens belonging to the Laeliinae subtribe, the presence of calcium oxalate druses for each cell of the structure had not yet been reported. To the best of our knowledge, this study is the first reporting the occurrence of druses in the suspensor, or more generally, in orchid seeds. In fact, such crystal conglomerates, according to Prychid and Rudall [44], are commonly found in various dicotyledons tissues but are more rarely observed in monocotyledons; concerning family Orchidaceae, druses have been recorded only in vegetative portions of Epidendrae and Dendrobieae [45,46], Arethusae [46], and in the >23% of the Oncidiinae [47].

Lee et al. [48], studying the suspensor of *Paphiopedilum delenatii*, observed that there was no cuticular material in the cell walls of the structure; considering the morphological traits of the transfer cell, they finally hypothesized that the suspensor was responsible for nutrient uptake for the embryo. Later, the role of embryo suspensor in nutrition has been confirmed [49,50]. In addition, Volk et al. [51] demonstrated that the deposition of druses in idioblasts is a dynamic process and, when Ca availability is reduced, cells could dissolve the crystals metabolizing CaOx to non-toxic compounds. According to these authors and considering our observations, it is possible that the suspensor possesses storage potential and, while providing a channel for nutrients conduction to embryo, could store the excess of calcium coming from the environment or could receive it as a byproduct of seed metabolism. Calcium oxalate, on the other hand, has many functional roles also for fungi, such as metal detoxification or increasing plant susceptibility to fungal infection, acting as an electron donor in lignocellulose degradation [52] and in the reduction of certain metals [53]. Oxalate, indeed, plays a unique role in lignocellulose degradation by basidiomycetes, acting as a low molecular mass agent initiating decay and as a potential electron donor for lignin-peroxidase catalyzed reduction [54–56]. This is particularly interesting because fungal colonization of orchid seeds by orchid mycorrhizal fungi (belonging to the Basidiomycota) occurs through the suspensor [57–60]. It is tempting to hypothesize that the druses have evolutionarily assumed the role of ready-to-use oxalate reserves available to the symbiont fungi to favor the degradation of the lignocellulose present in the seed coat [61] and their settlement as symbionts. It should be remembered that, in mycorrhizal fungi, the energy balance is very important [22] and even more so in orchid mycorrhizal fungi, where the debate in the scientific community is still ongoing [22,62,63]. Possibly supporting this hypothesis, Miura et al. [64] found that the presence of seed coat enhances seed germination with symbiotic fungi and protects the embryo against the attack of non-symbiotic fungi by restricting the invasion of their fungal hyphae. Indeed, the seed coat is a barrier against fungi that do not possess enzymes capable of degrading lignocellulose, making a sort of selection at the entrance.

However, as the druses deposition was recorded starting from the first two cells after the elongated pair and all along the suspensor (see Figure 3a,b), we cannot exclude that its detachment from the developing embryo in the early stages of germination might be a fast way to remove the excess of calcium oxalate. This hypothesis agrees to that discussed by Paiva [65], who concluded that the formation of CaOx crystals in portions that will be discarded allows the excretion of calcium, because plants lack excretory systems.

Germination was successful on all the media tested, but 1/2 MS was the best and is therefore suggested for *C. purpurata* propagation. Germination occurred also by using the fungal strain MUT4178 of *Tulasnella calospora* but with a lower percentage if compared to

the above-mentioned asymbiotic medium. Almeida et al. [26] indicated Tulasnellaceae (including *T. calospora*) as the main possible fungal symbionts for this orchid group; however, our results, which report the first attempt of symbiotic germination for *C. purpurata* (to the best of our knowledge) may indicate that the fungal strain used, isolated from an European terrestrial orchid, might not be the most appropriate or the dominant symbiont for this species, even if we demonstrated its colonization of roots (see also Adamo et al. [27]).

The subsequent development of seedlings grown *in vitro* allowed characterization of their morphology. According to the literature, this is the first study providing morphological information regarding *in vitro* seedlings of *C. purpurata*, because Gallo et al. [18] worked on the same species but in earlier stages of protocorm development, while Silva Júnior et al. [17] compared the anatomy of roots and leaves from plants treated with different urea concentrations.

We observed idioblasts containing raphides in both root and leaf tissues, adding information regarding their presence in the early development stages of seedlings. The occurrence, distribution, and morphology of idioblasts containing raphides have been reported by various authors who studied the vegetative tissues of most of the analyzed orchid subtribes [66] (and references therein). Stern and Carlsward [67] carried out a comparative study concerning vegetative portions of members from Laeliinae, and found that crystalliferous idioblasts, circular in transverse section and saccate in the longitudinal one, are present ubiquitously in the mesophyll of all the examined taxa. In general, the role of raphides for plant tissues also needs to be clarified. As recently revised by Paiva [65], various hypotheses about the functions of raphides in plant tissues have been indeed formulated; they could help in regulating calcium levels [51] or constitute a CO_2 source for photosynthesis in some species [68]. Tulyananda and Nilsen [69] stated that idioblasts spread in thin leaves of epiphytic species (in this case, *Rhododendron* sp.) act as buffering agents that significantly affect leaf-lamina water relations. As previously proposed by several authors and recently revised by Konno et al. [70], raphides could play a defensive role against herbivory.

Some of the anatomical characters observed in the roots and leaves of the studied species are typical to those of epiphytic orchid habit.

Root presented a 2–3 layered velamen, showing helical cell wall thickenings. Such a restricted number of velamen layers is characteristic of species belonging to relatively humid habitats [71]. Velamen avoids the loss of moisture from roots, speeds up water absorption, and provides protection against mechanical stresses and ultraviolet radiations [72,73]. On the other hand, helical thickenings in the velamen cells have the function to improve the stability and efficiency of this tissue for water absorption by the root and its retention in dry conditions in epiphytic orchids [74]. Passage cells constitute a channel for the selective transit of nutrients and water, and for the entrance of mychorrhizal fungi [71,75,76]. Tilosomes, branched lignified wall ingrowths on the internal periclinal wall of the endovelamen cells, are typically positioned near passage cells [77,78]. These structures, according to Kedrovski and Sajo [78], increase symplastic connections and improve water and solutes transport between external and internal environments in young tissues, while they steer solutes to passage cells in mature tissues. Stern and Carlsward [67] stated that there are no tilosomes in *Cattleya* but annotated their occurrence in other species that have been moved in this genus in times after their work, i.e., *Sophronitis sincorana* (now *C. sincorana*) and *Laelia anceps* (now *C. anceps*). Our observation confirms the fact that tilosomes are also present in *Cattleya* genus.

The monolayered exodermis with large elements with ∩-thickened cell walls is also typical of epiphytic orchids. This structure prevents transpiration and water loss from the cortex [71].

Taken together, the characteristics of exodermis, the number of velamen layers, the occurrence and orientation of thickenings in velamen cell walls, and the presence of tilosomes possess taxonomical value according to Porembsky and Barthlott [77]. To classify orchid roots, the latter authors indeed considered combinations of the aforementioned

characters, proposing 12 different syndromes; the complex of features showed by our study species resembles that of *Vanda*-type.

A high presence of chloroplasts was recorded in the cortex. This aspect is well known for Laeliinae [67] and in general for epiphytic orchids; many species have evolved photosynthetic roots to increase photosynthetic areas and consequently carbon gain [72,79,80].

The variable shape and the presence of thickened birefringent cell walls of cortex elements agree with what has been reported for other Laeliinae members by Stern and Carlsward [67]. Additionally, both endodermis and pericycle cells show characteristics already described by the latter authors (similar in dimension and O-thickened); however, contrary to what has been observed in general for Laeliinae, we recognized O-thickenings in correspondence of xylem rays instead of phloem ones, and protoxylematic elements in the pericycle. Lignin deposition in other elements of the central cylinder and endodermal cell wall thickenings may play similar functions to those of velamen and exodermis (prevention of water loss) and constitute another adaptative trait to tropical habitat conditions [71].

The number of xylem arches (10) falls in the range of 7–24, reported for this group of orchids [67]. Gallo et al. [18] recorded a triarch-tetrarch actynostele in developing protocorms, but it should be remembered that the organization of the vascular system undergoes changes during plant development [81].

Additionally, leaf characteristics are in line with the ecological adaptations of *C. purpurata* to a tropical environment. The roughness and thickness of *C. purpurata* leaf cuticles are consistent with characters and data previously annotated for Laeliinae [67]. The cuticle, relatively thick if compared to other orchid species [82], plays an ecological role in transpiration reduction and in drought resistance [83–85]. Similar considerations could be drawn for stomata dimensions, as small stomata are known to be quicker to close and then are more tolerant to dry conditions [84].

The stomatal apparatus is tetracytic, as noted in most Laeliinae; however, while a reniform shape was described for all the other taxa [66,67], the C-shaped guard cells that we observed were previously reported only for *Cattleya sincorana* (syn. *Sophronitis sincorana*). This latter record can be useful to corroborate taxonomical information regarding the studied species.

The data regarding the discontinuous hypodermis, the homogeneous mesophyll, and the dorsiventral one in correspondence of the central rib, together with the characteristics of vascular system, agree to what is generally known for Laeliinae [67]. On the other hand, the presence of transversally developed elements, which constitute anastomoses between veins in the mesophyll, was less frequently annotated in the subtribe. It has been proposed that they provide mechanical stability and discourage herbivory [86]. The occurrence of transverse veins in the mesophyll of *C. purpurata* is in line with previous findings for the genus *Cattleya* [87].

5. Conclusions

In conclusion, we compared germination on different media and, for the first time, attempted symbiotic germination for *Cattleya purpurata*, demonstrating the potential use of Tulasnellaceae, which otherwise need to be refined, looking for the best symbiotic fungal partner because germination percentage was lower than that obtained on some asymbiotic media. Furthermore, we characterized its seed and seedling morphology, providing new insights into the knowledge of this endangered species. Seedling morphology was comparable with other congeneric species, showing typical characteristics of epiphytic orchids such as root velamen with 2–3 cells layers. We confirmed the presence of tilosomes in roots in this orchid genus and observed stomata with C-shaped guard cells in leaves, which are unusually found in *Cattleya* species. Interestingly, we pointed out the presence of calcium oxalate crystals, both in idioblasts as raphides (in roots and leaves) and in the seed suspensor as druzes. It would be intriguing, in the future, to see if other orchid species actually accumulate these crystals in the suspensor and if they have a nutritional/functional role in the orchid symbiosis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7110480/s1, Figure S1: Correlation between the volume of the embryo and the seed analyzed by means of the cor.test( ) function using the Spearman's rank correlation coefficient rho, Figure S2: Main elements present in crystals determined by SEM-EDS spectrum. The analysis showed a chemical composition typically obtained for calcium oxalate, with carbon (C), oxygen (O) and calcium (Ca) peaks.

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Setting Up a Lab-Scale Pilot Plant to Study the New Growing System (NGS®) for Leafy Vegetable and Culinary Herb Growth

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Abstract: New cultural techniques have been developed to improve the yield and raw material quality at harvest, and enhance the postharvest shelf life, by standardizing the growing system. Among the different Soilless Cultivation Systems, the New Growing System (NGS®) is a closed-recirculating system that was designed for open fields and protected cultivations. The aim of this work was to investigate the structural setting of the system and its functioning to harness the full potentiality of NGS®. A lab-scale pilot plant (LSPP) was designed with NGS® technology and the technical aspects have been set up to have a standardized and reproducible growing system. The trials were conducted on growing mature-leaf vegetable species; that is, on both head and multi-leaf vegetables, and on culinary herbs at high plant densities. Positive yield results were found for culinary herbs and leafy vegetables. Mints showed high yields for the two re-growths carried out after the first harvest. The LSPP can also be used in a series of reliable experiments and enable researches to test several species, substrates, hydroponic nutrient solutions, and fertigation scheduling.

Keywords: growing efficiency; hydroponic nutrient solution; raw material standardization; soilless culture system; system design

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1. Introduction

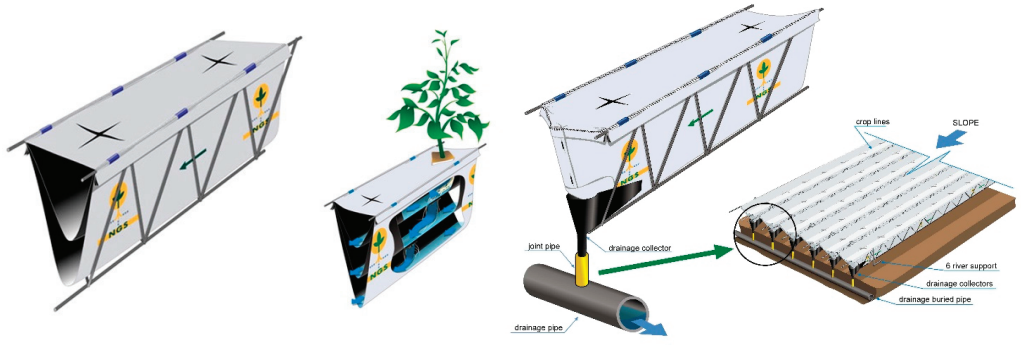
In crop production, soilless culture systems (SCS) encompass all systems that include plant cultivation in which the supply of water and minerals is carried out by means of a hydroponic nutrient solution (HNS), with or without a growing medium [1]. SCS have been introduced for protected crops for many reasons, including the necessity of: (a) avoiding soil usage to reduce several drawbacks (e.g., soil exhaustion, soil-borne diseases, secondary salinization, crop rotation); (b) improving the control of growth conditions (e.g., temperature and aeration of the root zone, water and nutrient distribution); (c) reducing the amount of labor needed [2,3]. SCS have been developed to increase yield and guarantee year-round availability, as well as to enhance the food quality, while assuring safety and extending the shelf life of the product. Several research areas have been developed regarding innovative SCS technologies based on the nutrient film technique (NFT). The mobile gully system (MGS), a highly automated NFT system developed by Hortiplan N.V. (Sint-Katelijne-Waver, Antwerp, Belgium) and the New Growing System (NGS®) developed by New Growing System S.L. (Pulpí, Almería, Spain), a modified NFT system, are two examples of innovative SCS technologies.

NGS® (patent no. 2.221.636/7) is a patented recirculating system that was designed for open field cultivation and introduced in several countries for a total commercial area estimated as ≈100 ha in 2005 [4]. No track record of updates on the number of acreages has been found ever since. NGS® was first designed as a fixed system for open-field

lettuce cultures in arid and semiarid areas, with the primary objective of rationalizing the use of water for horticulture production (www.ngsystem.com (accessed on 3 Februray 2021)) (Figure 1a). The system was later developed for use under cover and with a mobile system to automate operations and exploit high plant density. Owing to its differently constructed parts, NGS® can be adapted for smallholding farms (Figure 1b) or large advanced farms (Figure 1c).

Figure 1. (**a**): View of the New Growing System (NGS®) used commercially in open fields in Pulpí, Almería, Spain. (**b**): NGS® adopted on a smallholding farm. (**c**): View of a fully automated and mobile NGS® adopted on a large farm.

Because of its closed-recirculating system, NGS® allows the input control (e.g., water, fertilizers, chemicals, farm labor) at each moment of the cycle, as well as drainage reuse and wastage reduction [5]. In NGS®, plant roots grow homogeneously in a multilevel system, avoiding the obstruction of the HNS flow, and promoting root aeration [4] (Figure 2a).



(**a**) (**b**)

Figure 2. (**a**): View of the multi-channel film that is used to create separate channels for the flow of the hydroponic nutrient solution (HNS) and root development. Two channels are created for short crop cycle species, such as lettuce (**left**), while three channels are created for long cycle crop species, such as tomato, to allow root growth expansion (**right**). (**b**): View of the NGS® drainage collection system (courtesy of www.ngsystem.com (accessed on 5 September 2020)).

The multilevel system is made of a multi-channel film to favor the HNS flow as well as root growth and extension. The upper channel has lateral cuts designed to conduct the HNS to the lower layer when the root mass of the plants is obstructing the channel and to create small cascades, thus favoring HNS oxygenation. Multi-channel cuts on the inside direct the growing roots into the lower layer, thus avoiding a "bunged" effect of the HNS. The HNS flows into the channel in drip lines reaching to the end of the line by pressure and gravity of the multi-channel trough inside, which has a uniform slope (2%). The particular structure of the internal NGS® channel improves plant root oxygenation even when the channels are long. After watering and feeding the crop rooting system, all the drained HNS is collected in a drainage tank or cistern (Figure 2b).

NGS® achieves high planting densities and several growing cycles per year [6]. Furthermore, planting and harvesting operations can be carried out simultaneously in the fully automated system. The automation of the system can be managed according to the configuration of the space in the open field or greenhouse and the multiple sets of growing lines.

Like all the other SCS that do not have overhead irrigation and fertigation systems, NGS® reduces microbial contamination and avoids the presence of soil and chemical residues on plants, thus allowing softer washing procedures and sanitation processes, which in turn results in less stress for the leaf tissues [6,7]. Although NGS® has been used commercially for several years, there is a lack of NGS® literature on plant growth and standardization of the system in greenhouses, productivity, use of water, oxygen and nutrients, as well as on the biochemical composition of the raw material at harvest. Some smallholding farms have adapted the system to the local conditions (Contini, M., personal information), but the few experiments conducted using the NGS® are related to cultivation in the arid area of the Almería province (Spain) only and not with the specific purpose of studying the system [4–6]. Therefore, detailed investigations are needed to offer the growers technical information on NGS® management, to obtain Good Agricultural Practices, input utilization for single species and local vegetable crops, and to explore the eventual drawbacks of the system.

Having previously established and tested a lab-scale pilot plant (LSPP) in two greenhouses with another SCS, the floating growing system [8], a second LSPP was designed and built in order to have a representative and reliable experimental system. The LSPP based on NGS® was fully designed and then developed, equipped, and used for experimental purposes. NGS®-LSPP is the first experimental system based on the NGS® technology to be used in a systematic approach and adapted to research needs. The LSPP design and setting were achieved by investigating and studying each structural part of the system and its functioning to harness its full potentiality, in terms of yield, resource use and economic efficiencies. After the evaluations performed on the NGS®, the LSPP was designed as a scaled version of a commercial greenhouse growing system and the technical aspects were improved and standardized. The sizing of the LSPP was studied to cope with the necessity of both obtaining a reliable amount of data and assuring the scientific accuracy of each trial, to enhance and implement each trial progressively.

The LSPP is located in a typical continental climate region, the Po Valley in the North of Italy, where several thousands of hectares of leafy vegetables are used to produce raw material for the fresh vegetable market and fresh-cut industry. The LSPP will thus serve as a reference for the region and will be transferable to other growing conditions, such as in commercial scale or for other species. Consequently, NGS® could be explored in non-arid regions or those with a continental climate for water saving purposes. Having sound experimental facilities allows several environmental factors that affect plant growth to be investigated. This can be achieved by equipping the LSPP to test individual factors at the lab-scale and later on at the industrial scale. The efficiency of the NGS®-LSPP implementation was tested on growing mature-leaf vegetable species; that is, both head and multi-leaf vegetables, and on culinary herbs.

2. Materials and Methods

2.1. Design of the NGS®-LSPP

2.1.1. Greenhouse Setting

The NGS® pilot plant was set up in a protected environment in the Experimental Center of the Department of Agricultural, Forest and Food Sciences (DISAFA) (44°53′11.67″ N; 7°41′7.00″ E-231 m a.s.l.) in Tetti Frati, Carmagnola (TO), Italy. The greenhouse available in the center used for the setting of the LSPP is equipped with automatically controlled heating systems and pipelines that homogeneously distribute hot air, thus avoiding the formation of a temperature gradient. The greenhouse is equipped with an automatically controlled opening system to provide ventilation. During the warm seasons, the greenhouse is cov-

ered with non-movable black shading systems, with a 50%-shade cloth. The greenhouse is equipped with water distribution systems, which allow the HNS to be prepared directly in the same place where the experiments are carried out. A plastic greenhouse, equipped with an automatically controlled overhead irrigation system, is available and used as a nursery for the following trials in the LSPP. The nursery plastic greenhouse area is ≈210 m^2 and contains four benches for a total bench area of ≈65 m^2. The greenhouse in which the NGS® has been installed has an internal area of ≈160 m^2.

2.1.2. Mobile System and Frame

The NGS® was installed by fastening five steel bars (0.15 m wide × 0.05 m high × 7.00 m long) to the concrete floor, perpendicular to the greenhouse entrances (Figure 3a).

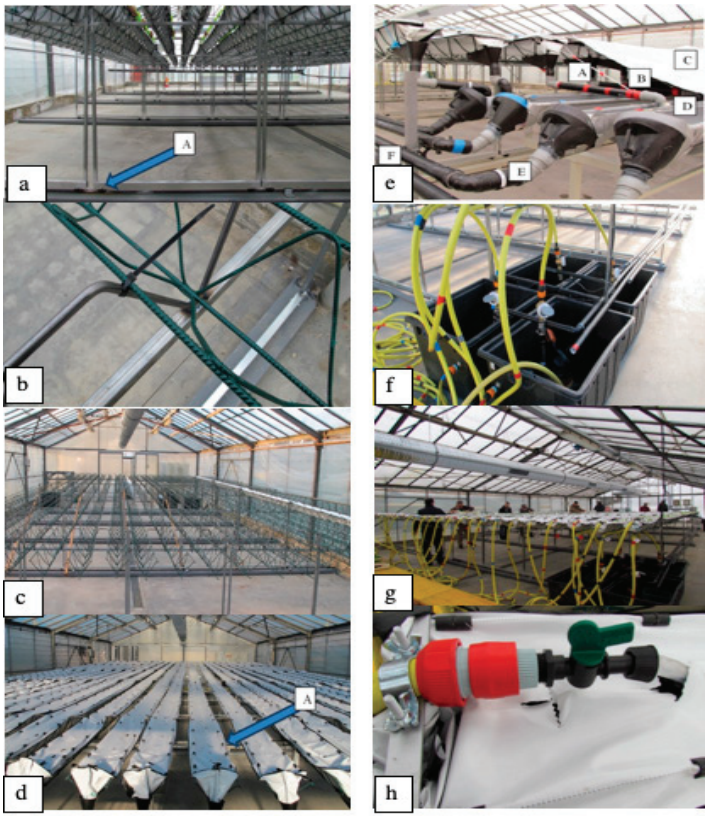


Figure 3. (**a**): View of the NGS® mobile supporting elements; (A) steel rail which allows lateral movement of the transversal support. (**b**): Details of the frame fixed to the transversal support. (**c**): View of the 24 NGS® lines under preparation. (**d**): View of the 24 NGS® lines equipped with the multi-channel film; (A) plastic clips used to fix the multi-channel film to the frame. (**e**): Drainage collection system of the 24 NGS® lines: (A) plastic drainage funnel, elbow and plastic drainage pipe that collect the HNS from the line; (B) elbow that directs the HNS drained to the gutter; (C) plastic film used to reduce the heating effect and prevent both evaporation and algae formation; (D) gutter that collects the HNS drained from six pre-assigned lines; (E) funnel, pipe and elbow that collect the HNS drained from the gutter; (F) plastic drainage connection pipe that returns the HNS drained to the drainage tank. (**f**): View of the four drainage tanks, each of which receives the HNS drained from six lines. (**g**): View of the hosepipes that convey the HNS to the NGS® lines. (**h**): Details of the HNS distribution system attached to the multi-channel film.

A steel rail (0.018 m outer diameter) was welded onto each steel bar to hold transversal supports. Each steel rail holds six 1-m wide transversal supports, for a total of 30 transversal supports in the greenhouse. The transversal supports were mounted onto wheels. The first transversal support of the row of each steel rail was fixed to allow the plastic drainage pipes to be anchored (see the Section 2.1.4) and the clutter to be reduced. The remaining five transversal supports can be moved laterally for 1 m by hand (Figure 3a, letter A). The possibility of moving the transversal support favors the passage and work of the employees between the growing lines. All the transversal supports are packed during plant growth to save space in the greenhouse and only a lateral corridor is viable on the peripheral sides. The top of the transversal support is an iron bar that has been shaped to fasten the steel frames necessary to hold the multi-channel film. Each transversal support has four perpendicular steel frames, for a total of 24 steel frames (Figure 3b). Each of the 24 lines is 12 m long, for a total length of ≈288 m (Figure 3c).

2.1.3. Multi-Channel Film and Drainage Funnel

The frame set up in the LSPP is 0.14 m wide and holds a multi-channel polyethylene film, which is white on the outside to reflect the light and black on the inside to avoid algae growth (Figure 3d). The multi-channel film is clamped to the metal frame structure with plastic clips (Figure 3d, letter A). The multi-channel film, given by the producer with a life span of three to five years, is 0.20 m wide; that is, 0.06 m wider than the frame to avoid the base surface covering the frame from being stretched too much. The multi-channel film used has three interconnected layers (Figure 2a, left drawing). The base of the upper layer has holes every 0.10 m, which were obtained from a cross cutting of 0.055 m × 0.055 m. The holes are suitable for holding transplants grown in peat pressed cubes or transplant plugs, the two types of transplants mainly used in the system, depending on the species and season.

At the end of each of the 24 lines (Figure 3e), plastic drainage funnels, elbows and plastic drainage pipes were fixed to direct the drainage of HNS into plastic gutters (Figure 3e, letter A).

Other elbows have been fixed to the end of the drainage pipes to avoid accidental movements of the system and the HNS from flowing into the wrong gutter (Figure 3e, letter B). A plastic film (white on the upper side and black of the lower side) was used to cover the drainage funnels, the drainage pipes and the gutters to reduce the heating effect and prevent both evaporation and algae formation (Figure 3e, letter C).

2.1.4. Gutter and Closed-Recirculating System

Four oval open plastic gutters (0.12 m wide × 0.08 m high × 7.00 m long) were installed on a mobile support to collect the HNS flowing from the six lines (Figure 3e, letter D). The setting was planned for experimental purposes to be able to test four levels of each treatment at the same time replicated in three blocks and randomly assigned at the beginning of each experiment. The HNS drainage flows from each gutter into a plastic funnel, a pipe and an elbow (Figure 3e, letter E) and then to a 12-m long connecting plastic drainage pipe anchored to the fixed transversal support (Figure 3e, letter F). The connecting drainage pipe ends up in a 160-L volume plastic drainage tank (0.52 m × 0.74 m upper and 0.47 m × 0.69 m lower sides, respectively, 0.46 m high) (Figure 3f). Each drainage tank can contain one of the four possible levels. The drainage tanks are also covered by the same type of plastic film used to cover the drainage funnels, the drainage pipes and the gutters. Each drainage tank is equipped with a nylon net mash filter to block peat, roots, and organic matter that could be ripped away by the HNS flow. The net filter use is the result of the need to avoid potential damage to the submersible pump contained in each tank (QSB-JH-25027, 230 V/50 Hz; Techtop Industries, Inc., Alpharetta, GA, USA). Each submersible pump is connected to six lines by a hosepipe to homogeneously convey the HNS into the system (Figure 3g). The hosepipes all have the same length to avoid differences in the HNS flow timing in the LSPP. Two ball valves were placed on each pump

to better standardize the HNS flow and regulate the pressure: one at the bottom of the pump to regulate the HNS amount discharged into the tank, and the other at the top of the pump to regulate the HNS amount pumped into the system. All of the pumps can be scheduled intermittently by a control unit, according to the crop requirements. This distribution system is fixed to the frame by means of steel pipe clamps. Tap valves were placed between the hosepipes and the multi-channel films. A 0.10 m Cristal tube was fixed after the valve to correctly direct the HNS into the NGS® lines (Figure 3h). The valves can be regulated, according to the experimental needs, to distribute a specific amount of HNS per minute along each line. The HNS flow volume is recorded by flow meters placed after each pump and before each drainage tank. When the system is off, each drainage tank contains ≈ 100 L of HNS, which is necessary to keep the pump submerged.

Each drainage tank has been equipped with a floating sensor to detect the level of HNS in the tank. The sensor sends a signal to the reservoir tank, which indicates the necessity of refilling the HSN consumed by the plants with new HNS. The new HNS enters into the four drainage tanks via hosepipes from four 100-L stainless steel cylindrical reservoirs (0.74 m high and 0.41 m o.d.). A graduated vessel, made of a Cristal tube, is positioned outside each reservoir and is connected to the inside; this indicates the level of HNS. The decision to position the graduated vessel outside each reservoir was made to: (a) avoid the use of a floating indicator in the reservoir; (b) easily control the HNS level in the reservoir without the need to use steps or stairs; (c) avoid opening the reservoir cover, thus, preventing both evaporation and algae formation.

The LSPP has been set up to exploit gravity to create a closed-recirculating system: the slope of the lines, of the gutters and the connecting drainage pipes has been set to 1%. The reservoirs are positioned at a height of ≈1.30 m from the ground to fill the drainage tanks by gravity. Pumps are only used at the beginning of the closed-recirculating system to move the HNS from the drainage tanks into the NGS® lines. This set up has been created to favor the economic efficiency and the sustainability of the system in terms of energy consumption. Additional reservoirs are available in the greenhouse and can also be set up and connected to the pre-installed reservoirs to allow a greater amount of new HNS, particularly in warm periods. The tanks and reservoirs contain submerged pumps to stir the HNS at a scheduled frequency and intensity, thus avoiding salt deposition and favoring aeration. All of the pumps can work at the same time.

It is known that the oxygen concentration in the HNS is a limiting factor for plant growth and it can cause hypoxia or even anoxia [4,9,10]. The cascades originating from the multi-channel film, the intermittent flow of the HNS, the stirring effect of the pumps in the tanks and in the reservoirs have been set up to help oxygenate the plant roots that are hanging in the air. HNS oxygenation allows plant root health to be maintained and enhances plant quality while obtaining high yields [11]. The design of the LSPP unit and flow is presented in Figure 4.

2.1.5. Measurements

Several measurements can be carried out during LSPP utilization to monitor and control its functioning as well as the growth conditions. Temperature and relative humidity can be measured constantly: data loggers for air and HNS temperatures and air relative humidity recordings were later added to the system to continuously monitor and record air and HNS temperatures and the water temperature in each drainage tank of the HNS (Misol DS102 thermo-hygrometer; Fine Offset Electronics Co., Ltd., Shenzhen, China and Elitech RC-4 thermometer, Elitech Inc., Milpitas, CA, USA). The photosynthetic photon flux (PPF) can be measured on the top of the NGS® lines using a data logger (LI-1000 DataLogger; LI-COR, Inc., Lincoln, NE, USA) equipped with a sensor (Quantum sensor LI-190SA; LI-COR, Inc., Lincoln, NE, USA). The pH, electrical conductivity (EC), and temperature can be measured in the HNS by means of a Waterproof CyberScan PC 650 (Eutech Instruments Pte Ltd., Singapore), equipped with a submersible pH electrode (ECFC7252203B) and an EC/temperature probe (CONSEN9203J). In our experiments, the pH of the HNS was

monitored continuously to be kept close to 5.5, while the EC was kept between 2000 and 2500 $\mu S \cdot cm^{-1}$ using an acidic or basic solution to neutralize the salts, thus, avoiding immobilization or precipitation of the salts themselves, and increasing their availability for plants [12,13].The acid and basic solution are added in the drainage tanks when in their steady state. The oxygen content dissolved in the HNS can be measured by means of an oximeter (YSI 550A; YSI, Inc., Yellow Springs, OH, USA). The prepared HNS volume that is added to the reservoirs is recorded during the growing cycle (Figure 5), as well as all the daily activities.

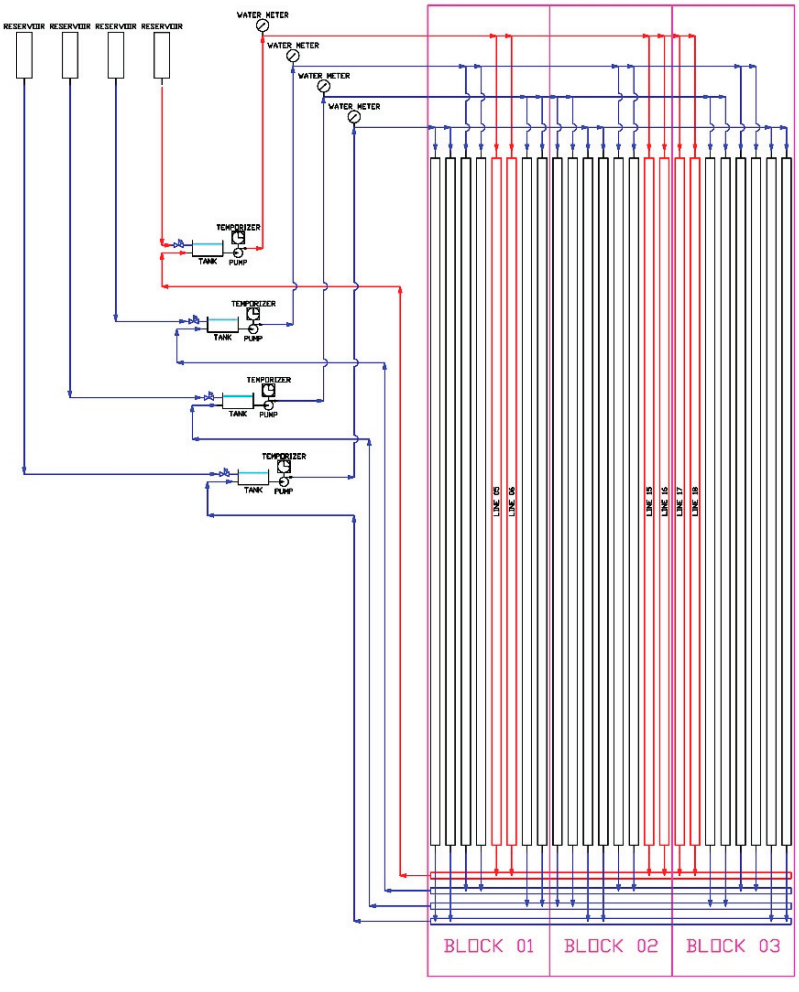


Figure 4. Scheme of the lab-scale pilot plant (LSPP). The blue circuit refers to the HNS flow. The red path represents a single treatment; the specific path was chosen randomly for representation purposes. The blue rectangles are the gutters. The black rectangles are the NGS® lines.

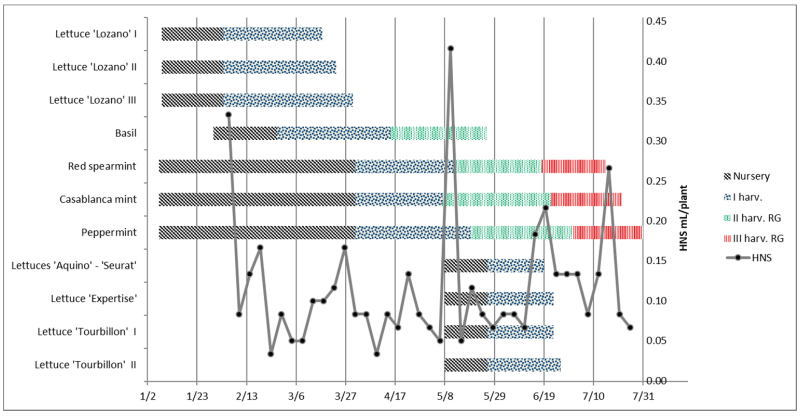


Figure 5. Growing cycle of the leafy vegetables and culinary herbs tested during the experimental period. The bars with black diagonal lines indicate the nursery stage. The bars with blue squares indicate the NGS® growing cycle-I harvest. The bars with green points indicate the NGS® growing cycle-II harvest, regrowth (RG). The bar with red vertical lines indicates the NGS® growing cycle-III harvest, RG. The grey line represents the HNS flow per plant added to the system during the growing period (HNS mL/plant, indicated by the axis in the right).

2.2. Plant Growth

The HNS composition and concentration used in the experiments were defined on the basis of the results obtained by the Vegetable Crops & Medicinal and Aromatic Plants-VEGMAP research group [8,10,14,15]. The HNS was prepared by dissolving salts (purity > 98%) in tap water with a known salt composition. The tap water has an EC of 440 $\mu S \cdot cm^{-1}$, with a pH of 7.5 and 24 °f of hardness. Elements present in the tap water were considered in preparing the HNS. The HNS was composed of: 6N-2P-6K, 2 Mg, and 2.5 Ca (all in $mmol \cdot L^{-1}$), with a ratio of 40/60 $N\text{-}NO_3^-/N\text{-}NH_4^+$. The high ammonium level is used in winter to keep low nitrate accumulation in plants, while in summer crops the ratio is reverted. Microelements, Oligogreen (0.03 g L^{-1}) and Kelagreen Fe (0.03 g L^{-1}) (Green Has Italia S.p.a., Canale d'Alba (CN), Italy), were added to the HNS. In order to test the pilot plant for the future plant growth experiments, the following species were investigated to verify the suitability of LSPP to grow mature-leaf vegetable species; that is, both head and multi-leaf vegetables, and culinary herbs: lettuce (*Lactuca sativa* L.) 'Lozano', Salanova® 'Aquino', Salanova® 'Seurat', Salanova® 'Expertise', 'Tourbillon'; basil (*Ocimum basilicum* L.) 'Superbo'; red spearmint (*Mentha spicata* L. var. rubra); Casablanca mint (*Mentha spicata* L. var. viridis); black peppermint (*Mentha* × *piperita* L. var. *officinalis* forma *rubescens* Camus).

The lettuces and basil were sown in peat press cubes and grown in a local nursery (Azienda Agricola Vivaistica Ricca Sebastiano, Carignano (TO), Italy) until the transplanting date. The peat cubes were prepared using a specific commercial peat-based horticultural medium (Brill 5; Gebr. Brill Substrate GmbH & Co. KG, Georgsdorf, Niedersachsen, Germany) and were 0.033 m × 0.033 m × 0.033 m. All of the mint species were propagated from cuttings from mother plants in 60-cell Styrofoam trays and then transplanted into pots (0.072 m × 0.072 m upper and 0.055 m × 0.055 m lower sides, respectively, 0.074 m high or 0.062 m × 0.062 m upper and 0.047 m × 0.047 m lower sides, respectively, 0.068 m high) using the Neuhaus Huminsubstrat N17 peat-based horticultural medium. Plantlets of mint species were grown in the nursery plastic greenhouse of the Experimental Center until the transplanting date. The plants were overhead irrigated for 1 min twice a day each day during growth, until transplanting. When the plants reached a suitable growth stage, which depended on the species, they were moved into the LSPP and planted at 0.20 m (lettuce) or 0.10 m apart (other species), depending on the species, reaching respectively a plant density of 20 or 40 plants per m^2. The total number of plants used per experiment

outlined in the three blocks was: ≈120 plants of 'Lozano' lettuce and of basil; ≈70 plants of red spearmint, of Casablanca mint, and of peppermint; ≈50 plants of 'Tourbillon' lettuce; ≈25 plants of 'Aquino' lettuce and of 'Seurat' lettuce; ≈10 plants of 'Expertise' lettuce. The various species were cultivated in LSPP in different seasons from February to July and adopting different harvesting practices, ranging from one-cut to regrowth (Figure 5). The Lozano and Tourbillon cultivar of lettuces were periodically harvested and sampled to measure the growth parameters until head maturity. The Aquino, Seurat, and Expertise cultivars of lettuces were sampled at harvest and then removed from the NGS®. The basil crop was allowed to regrow after the first canopy harvest to compare the one-harvest strategy with the two-harvest strategy, in terms of system efficiency. The three species of mint were allowed to regrow twice, thus leading to a total of three canopy harvests.

Harvesting took place when the plants reached the proper growth stage, depending on the species. The harvesting and raw material handling followed standard procedures throughout the trials to obtain replicable and comparable experiments, and timing efficiency of the sampling procedures in order to validate the pilot plant system. The tools used for harvesting were sanitized before use, and the crew took particular care of their hand sanitation, their personal clothes and their hygiene. Harvesting was conducted early in the morning to avoid the hottest hours of the day. Fresh mass production was obtained by harvesting all the raw materials produced in the LSPP. After harvest, the raw materials were immediately used for biometric determination. The leaf fresh weight (LFW) per plant was computed and the yield was measured. After drying at 60 °C to a constant weight, dry matter (DM) was calculated.

2.3. Statistical Design

The LSPP was set up to deal with three blocks per experiment. The blocks were planned according to the amount of light exposure due to the greenhouse orientation. The greenhouse is East-West-oriented and the blocks are on the Northside, center, and Southside, respectively. Each block includes eight lines, which allow up to eight possible treatments. Each line can accommodate 120 plants, if they are planted 0.10 m apart (e.g., basil, mint in the first experimental trials carried out) or 60, if they are planted 0.20 m apart (e.g., lettuce). The maximum number of plants that can be transplanted in the LSPP is ≈2880.

3. Results and Discussion

Moving from soil to soilless culture systems can increase water use efficiency, particularly in closed-loop systems with a recirculating water/nutrient solution that recollects the drain water for reuse [16,17]. A number of researches have reported that soilless culture systems (SCS) allow control of growth factors and clean leaf production, easing and shortening postharvest handling in process industries [18–20]. The most common hydroponic systems used for leafy crop production are NFT and DFT systems (DFT = Deep Flow Techniques, also called Floating Systems) [8,21–24], but little information is present in the literature about NGS® for leafy crop production. For these reasons, the structural setting, the technical features, and the functioning of NGS® were studied. The main advantages of the NFT systems are the absence of the growth substrate and the reuse of drainage [25], which allows water and fertilizer savings between 50% and 80%, compared to conventional cultivation systems (http://ngsystem.com/news (accessed on 15 January 2021)). In light of this, the use of NGS® avoids the negative impacts on the surrounding ecosystems as well as the contamination of groundwater used by humans [26]. An additional benefit is the great potential for automation to save on labour costs (planting and harvesting) and the opportunity to manage the optimal plant density during the crop cycle. NGS®, being a similar system to NFT, allows input control and improves the earliness of the crops; in addition, it can be used in a series of reliable experiments and enables researches to test several species, substrates, hydroponic nutrient solutions, and fertigation scheduling.

Plants of the *Asteraceae* and *Lamiaceae* families were used in this research to evaluate the efficiency of NGS®. Indeed, green vegetables like lettuce and herbs are well suited to hydroponic systems as they have low to medium nutritional requirements [27]. Positive plant growth results have been found in the NGS® system in terms of leaf fresh weight (LFW), leaf daily growth (LDG), yield, and dry matter (DM) (Table 1). Lettuce 'Lozano' showed high yields for the two re-growths carried out after the first sampling (35.62 g). In particular, the plant fresh weight was 42.89 g and 57.35 g at the second and at the third sampling, respectively. These data are in line with the leaf daily growth and yield per square meter. The dry matter content of lettuce was 6.36 g at the first sampling, 8.11 g at the second sampling and 7.62 g at the third sampling. In addition, Tourbillon lettuce at the second sampling exhibited higher leaf fresh weight (53.93 g). High values of dry matter of lettuce were confirmed by Selma and coauthors [6] who compared green and red lettuce grown in an open field in NGS® and in soil. Specifically, the authors found that, apart from a genotype influence on the quality parameters, NGS® led to better quality and safety than soil production.

The cultivation of mint in the NGS® system has been accomplished with the purpose of assaying an alternative cropping system to the traditional common agronomic practice for growing pepper mint in the region of the Experimental Center. The common practice is based on the production of mint in soil, with typically two harvests per year, one in early summer and one in late summer (Nicola et al., 2004). Mint plants grown in the NGS® system were harvested three times during the experimental period (Table 1), with the measured parameters differing in behavior between the harvests. The Red spearmint first harvest occurred after 42 days of growth, the second harvest occurred after 27 days of regrowth and the third harvest occurred 37 days after the second regrowth. Red spearmint leaf fresh weight, yield and dry matter increased over the regrowth, with values at the third harvest of 29.73 g/plant, 1189.39 g m^{-2} and 18.99%, respectively. A different behavior was observed in Casablanca mint plants. More specifically, the fresh weight increased at the second harvest (35.24 g/plant) and then decreased (24.46 g/plant) at the third harvest. Conversely, dry matter content increased over regrowth: 18.10% at the first harvest, 18.31% at the second harvest and 18.89% at the third harvest. The greatest yield was obtained in the second growth, but with the same LDG as the third growth. The fresh weight of Peppermint leaf increased at the second harvest (19.95 g/plant) compared to the biomass collected at the first harvest (17.05 g/plant). At the end of the experiment, a decrease in the leaf fresh weight was evident (11.62 g/plant). Pepper mint yield followed the same trend, with values of 681.85 gm^{-2}, 797.85 gm^{-2} and 464 gm^{-2} at the first, second and at the third harvests, respectively. Basil was allowed to regrow only once, leading to two harvests. The species is not adapted to continue regrowth in soilless culture due to risks of *Peronospora* sp. attacks. The results showed that at the first harvest the yield was 639.33 g m^{-2} and at the second it was 519.2 g m^{-2}, and the dry matter was 10.93% and 10.57%, respectively. In a paper by Walters and Currey [28], the authors quantified the productivity and characterized growth of basil cultivars grown in two hydroponic production systems. Thirty-five basil cultivars were chosen and grown into NFT or DFT systems for 3 weeks. The authors showed that there was no interaction between basil cultivars and hydroponic production systems, and the yield of basil was affected more by cultivar selection than hydroponic production system.

The higher oxygenation due to the particular structure of the internal NGS® channel is useful to increase the plant productivity. In this respect, Urrestarazu and coauthors [4] studied the oxygenation aspects of NGS® in tomato, cucumber and sweet pepper plants. The results showed that NGS® ensured better growing conditions compared to NFT.

Table 1. Distance between plants in the multi-channel (PD M-C), growth duration (GD) leaf fresh weight (LFW) per plant, leaf daily growth (LDG), yield per square meter, dry matter (DM) in the leafy vegetables and culinary herbs grown in the New Growing System (NGS®) using the lab-scale pilot plant. The LFW, Yield and DM of the lettuce 'Lozano'-I sampling; red spearmint-II harvest, regrowth; Casablanca mint-II harvest, regrowth are the means of ≈60 plants ± standard error (SE). The LFW, Yield and DM of the lettuce 'Lozano'-II sampling and III sampling are the means of ≈30 plants ± SE. The LFW, Yield and DM of basil-I harvest are the means of ≈120 plants ± SE. The LFW, Yield and DM of basil-II harvest, regrowth are the means of ≈100 plants ± SE. The LFW, Yield and DM of red spearmint-I harvest and III harvest, regrowth; Casablanca mint-I harvest and III harvest, regrowth; peppermint-The LFW, Yield and DM of peppermint-III harvest, regrowth are the means of ≈50 plants ± SE. The LFW, Yield and DM of the lettuces: 'Aquino'-'Seurat' and 'Tourbillon'-I sampling and II sampling are the means of 25 plants ± SE. The LFW, Yield and DM of the lettuce 'Expertise' are the means of 10 plants ± SE. Seeds provided by: [z] Rijk Zwaan; [y] Sais Sementi.

Species	PD M-C	GD	LFW	LDG	Yield	DM
	(m)	(days)	(g/plant)	(g/plant/d)	(g m^{-2})	(%)
Lettuce 'Lozano' [z]-I sampling	0.20	42	35.62 ± 1.61	0.72 ± 0.04	712.49 ± 32.16	6.36 ± 0.26
Lettuce 'Lozano' [z]-II sampling	0.20	48	42.89 ± 2.01	0.79 ± 0.04	857.74 ± 40.14	8.11 ± 0.18
Lettuce 'Lozano' [z]-III sampling	0.20	55	57.35 ± 2.41	0.95 ± 0.06	1147.03 ± 48.27	7.62 ± 0.10
Basil [y]-I harvest	0.10	48	15.98 ± 0.39	0.31 ± 0.01	639.33 ± 15.57	10.93 ± 0.21
Basil [y]-II harvest, regrowth	0.10	41	13.00 ± 0.33	0.29 ± 0.01	519.85 ± 13.33	10.57 ± 0.14
Red spearmint-I harvest	0.10	42	21.30 ± 2.37	0.39 ± 0.06	852.12 ± 94.94	17.30 ± 0.56
Red spearmint-II harvest, regrowth	0.10	37	34.77 ± 2.20	0.80 ± 0.06	1390.97 ± 87.90	16.26 ± 0.90
Red spearmint-III harvest, regrowth	0.10	27	29.73 ± 6.54	0.92 ± 0.24	1189.39 ± 261.52	18.99 ± 1.32
Casablanca mint-I harvest	0.10	37	15.61 ± 2.11	0.29 ± 0.06	624.25 ± 84.40	18.10 ± 0.43
Casablanca mint-II harvest, regrowth	0.10	46	35.24 ± 1.60	0.66 ± 0.03	1409.79 ± 63.82	18.31 ± 1.17
Casablanca mint-III harvest, regrowth	0.10	30	24.46 ± 2.83	0.65 ± 0.09	978.46 ± 113.00	18.89 ± 0.28
Peppermint-I harvest	0.10	49	17.05 ± 1.47	0.25 ± 0.03	681.85 ± 58.79	17.09 ± 0.59
Peppermint-II harvest, regrowth	0.10	43	19.95 ± 0.91	0.35 ± 0.02	797.85 ± 36.39	17.92 ± 0.67
Peppermint-III harvest, regrowth	0.10	30	11.62 ± 2.46	0.22 ± 0.08	464.99 ± 98.57	22.96 ± 1.46
Lettuces 'Aquino' [z]-'Seurat' [z]	0.20	24	56.42 ± 2.04	2.14 ± 0.09	1128.33 ± 40.82	7.45 ± 0.45
Lettuce 'Expertise' [z]	0.20	28	76.72 ± 7.39	2.56 ± 0.26	1534.33 ± 147.74	6.62 ± 0.13
Lettuce 'Tourbillon' [z]-I sampling	0.20	28	53.43 ± 0.73	1.73 ± 0.03	1068.53 ± 14.68	8.17 ± 0.37
Lettuce 'Tourbillon' [z]-II sampling	0.20	31	53.93 ± 1.50	1.58 ± 0.05	1078.60 ± 29.94	7.88 ± 0.24

Unfortunately, owing to the lack of information available in the literature on the biometric parameters of species grown in NGS®, an extensive comparison of the results obtained in the preliminary experiment with those of other experiments on NGS® was not possible. However, it is pivotal to point out that hydroponic production systems should be chosen based not only on plant yield but also on factors such as usability and input requirements.

4. Conclusions

NGS® technology is an innovative and versatile SCS that is suitable for growing plants as it promotes root aeration and prevents HNS-flow obstruction. Owing to the lack of literature and guidelines on NGS®, an LSPP based on NGS® technology was set up in a greenhouse in a mild continental climate to obtain a standardized, homogeneous, and representative experimental system. The LSPP was designed, set up and implemented to exploit the potentiality of the NGS® technology to reach technical and system improvements. The LSPP allows multiple treatments and replicates to be conducted to perform reliable statistical design and data analyses. The functioning of the LSPP was evaluated on both leafy vegetables and culinary herbs through preliminary quantitative analyses. The LSPP installed in the Experimental Center, because of its lab-scale size, could provide the basis for detailed investigations on the tested species and the research could then be extended to other species and agronomic factors. Future research will allow study of the system in detail, analyzing the potential advantages and drawbacks for using it for commercial purposes.

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Tree Planting Density and Canopy Position Affect 'Cerasuola' and 'Koroneiki' Olive Oil Quality

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Abstract: To maximize orchard production and tree crop efficiency, optimization of both maximum orchard light interception and radiation distribution within the tree canopy are important strategies. To study the influence of planting density and fruit position within the canopy on oil quality from 'Cerasuola' and 'Koroneiki' olive (*Olea europaea* L.), fruits were harvested from the upper and lower canopy layers of trees in hedgerow planting systems at two densities: High at 1000 trees ha^{-1} (HD) and Medium at 500 trees ha^{-1} (MD). Tree crop efficiency and fruit weight, water and fat content were measured together with olive oil standard quality parameters, phenolic and volatile composition. Fruits in the upper layers of the canopy always showed a higher maturity index, 6% more fat content, and 4% less water content than lower layers. Upper layers of HD trees showed the highest phenol content, whereas lower layers of MD trees showed the lowest phenol content (36% less than the upper layers of HD). HD trees showed the largest differences in fruit maturation, water and fat content between upper and lower canopy positions, increasing quality and oil yield variability at harvest. 'Koroneiki' showed more stable oils with a 28% higher MUFA/PUFA ratio and 12% higher phenol content than 'Cerasuola' oils. This study provides further evidence of the fact that cultivar, planting density, and canopy architecture may be strong determinants of olive oil yield and composition in hedgerow planting systems.

Keywords: *Olea europaea*; fat content; fatty acid profile; phenolic content; volatile compounds; hedgerow planting systems

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1. Introduction

Due to a global increase of olive oil consumption and labor costs many olive (*Olea europaea* L.) growers are moving towards the use of hedgerow super-intensive planting systems. Earlier bearing, increased yield, and a reduction of alternate bearing, as well as cost and facilitation of complete mechanization, have spurred adoption of new and super-intensive planting systems. Sicily represents an important center of olive oil production in the Mediterranean basin, where 'Cerasuola' is one of the most common olive cultivars [1]. The variety adapts well to poor soils, is drought-resistant, and provides excellent results under optimal nutritional conditions [2,3]. The oil content in the drupes of 'Cerasuola' is relatively high (20–25%), and, according to sensory evaluations, generally falls in the category of medium intensity fruitness along with the taste sensations of bitter, pungent, and sweet [3]. Recently, the international cultivar 'Koroneiki' has been introduced in Sicily. 'Koroneiki' is a cultivar of high vigor with an upright growth habit that partially satisfies

requirements for super-intensive planting systems. Its fruits are rather small, and the olive oil produced by 'Koroneiki' has excellent quality and fragrance, classified as a very fruity oil with green-apple notes, medium level aroma of leaves and grass, bitter and pungent. It is also astringent with a touch of almond, fig, and bark [4].

Due to its nutritional and health-promoting effects, the consumption of olive oil has been increasing worldwide, even in countries where it is not produced [5]. The nutritional and health promoting effects of olive oil have been associated with the optimal balance between saturated, mono, and polyunsaturated fatty acids as well as to minor components such as chlorophyll, polyphenols, and tocopherols [6]. Marketing of high quality olive oils is based on the chemical and sensory attributes, which are strongly affected by genotype, environment, fruit maturity at harvest, and their interaction among other factors [7–9]. Previous studies evidenced that high quality olive oil requires harvesting olive fruit at the optimum time [10–12]. The rate of oil synthesis and the length of the oil accumulation period can be responsible for the final oil content in the olive fruit [13,14].

High production costs, especially for harvest, have played a key role in the redesigning of olive orchards during the last 30 years [15]. For an optimal yield and maximum light interception, optimum planting density should be determined [16]. In addition to tree spacing, cultivar, climate, harvest method, tree training system, fertilization, irrigation management, and soil conditions should be properly considered. It was reported that along with the reduction of row spacing (ranging from 7 to 3 m), the management of orchard light interception should be taken into consideration [16–18]. Moreover, increasing tree planting density alters interception of solar radiation and distribution of radiation within the tree canopies during the orchard development [17,18]. This allows for managing the efficiency of solar radiation used for different processes including photosynthesis, flower bud formation, growth, and fruit quality. Jackson [17] indicated that to maximize orchard production and efficiency, both interception of maximum amount of radiation and optimization of the radiation distribution within the canopy are important factors. In olive trees, fruits located on the periphery of the canopy which exploited more solar radiation were bigger and with higher oil contents compared to fruits from internal parts of the canopy [19]. In 'Arbequina' hedgerows, fruits from the upper part of the canopy showed more advanced maturity and larger size. Furthermore, oil content increased by nearly 50% from lower to upper layers (Gómez-Del-Campo et al., 2009b). Hence, it can be concluded that intercepted radiation determines some of these differences, such as fruit size and oil content. In addition, differences in oil quality can result from rapid growth and early maturation in the upper layers of the tree canopy. Indeed, previous studies indicated that irradiance received in different hedgerow positions and orientations influenced fruit development and oil quality in olive. Fruits receiving more radiation showed the highest fruit weight, mesocarp oil content, maturity index, and total polyphenols in virgin olive oil [20]. Recently a study of 'Arbequina' also found that oil extracted from the upper layers presented higher concentration of oleuropein and ligstroside aglycone [12]. These compounds, besides having high antioxidant capacity, also contribute to the bitter and pungent flavor of the oil [21]. On the other hand, several studies reported that biomass production was directly related to canopy light interception in other fruit trees such as apple and peach [22–25]. In 'Arbequina' olive trees trained to 2.5–2.9-m hedgerows, the fatty acid composition of the extracted oils was significantly affected by the amount of intercepted light [26].

The aim of this study was to evaluate the influence of fruit position in the canopy and planting density on the production, fruit characteristics, and oil quality parameters such as phenol contents and volatile compounds from 'Koroneiki' and 'Cerasuola' olives. 'Koroneiki' was included as a reference cultivar since it is already implemented in super-intensive planting systems while 'Cerasuola' is a major Sicilian cultivar usually grown in traditional planting systems and appreciated for its quality oils.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Fruits were obtained from olive trees grown in an experimental field located in the southwest region of Sicily (37°31′ N, 13°03′ E, about 120 m a.s.l.). The orchard was planted in 2012 using 1-year-old self-rooted olive trees of the cultivars Cerasuola and Koroneiki. Trees of the two cultivars were planted in a single north–south row at two planting densities: 2 × 5 m (1000 trees/ha, HD) and 4 × 5 m (500 trees/ha, MD). The trees were pruned lightly during the first 5 years after planting and trained to free palmette, a two-dimensional tree shape, to facilitate mechanical harvesting. Two self-compensating in-line drippers per plant, delivering 16 L/h, were used for weekly irrigation, from July through mid-September. The total seasonal application rate was 640 and 320 m^3/ha/year, for HD and MD plantings, respectively. Fruit yield (kg/tree), yield efficiency (kg cm^{-2}), and yield (t ha^{-1}) were calculated for each tree as a biological replicate. All other measurements performed in the fruit and oil were determined separately in the upper and lower layers of the canopy.

2.2. Olive Harvest

On 15 November (for 'Cerasuola') and 17 November (for 'Koroneiki') of 2017, 28 trees for each cultivar were selected based on similarities for fruit load, number of branches and light distribution in canopy. To test the effect of the fruit position in the canopy fruits were harvested separately from the upper half of the canopy (1.5–3.0 m) or the lower half of the canopy (0–1.5 m). Fruits were hand harvested and placed in 1-ton bins for processing. Fruits were weighed and processed with a commercial two-phase mill (Toscana Enologica Mori-TEM) with a working capacity of 400 kg of olives/run. The oil extracted from each combination of factors (cultivar, planting density and canopy position) was subsequently weighed, and subsamples taken for chemical analyses.

2.3. Fruit Measurements

Fruits from upper and lower canopy layers at each planting density were assessed for fresh weight of 100 fruits (g) and maturity index based on skin and pulp pigmentation [27]. Fruit moisture content (% of fresh weight) and fat content (% of fresh weight) were determined by near infrared (NIR) measurements using an Olivia NIR analyzer (FOSS, Hillerød, Denmark). Percentage of black peel cover color was determined by digital image analysis as described in Farina et al. [28] using 32 photo replicates per cultivar (two per tree from 16 trees), each containing 45 fruits. Specifically, we used an algorithm that converts images from RGB to CIE (Commission Internationale de l'Eclairage) L*a*b* format, extracts the fruit from the image (removing the image background), and quantifies color characteristics as the weighed distance of each pixel in the image from a reference sample (darkest area interactively chosen from a well colored fruit). A green–red threshold algorithm was added to the previous procedure to obtain a separation of the total fruit area (in number of pixels) into two subregions: black color (closer to red) and ground color (closer to green). The pixel ratio between the red-colored area and total fruit area was used to quantify the percentage of black peel color (or degree of veraison).

2.4. Oil Quality Traits

Free acidity (% of oleic acid) and peroxide value (mEq O_2 kg^{-1}) were measured according to the European Union standard methods (UE, 1989/2003 modifying the ECC 2568/91). According to the limits established by the International Olive Oil Council [29] for free acidity, peroxide value, and organoleptic characteristics, all oils studied were classified as extra virgin olive oils (EVOOs) (Table 1). Chlorophyll and carotenoid contents were measured colorimetrically using a Beckman DU 640 UV spectrophotometer at 476 and 670 nm, as described by Mineo et al. [30]. The chlorophyll and carotenoid contents were expressed as mg of chlorophyll a and β-carotene per kg of oil, respectively.

Table 1. Main olive oil quality traits. Average and standard deviation (SD) of all olive oil samples (n = 24). Limits for classification of extra-virgin olive oil by the International Olive Council.

Main Quality Traits	Limits Described in	Mean ± SD
	IOOC/T.15/NC No 3/Rev. 11	
Free acidity (%m/m expressed in oleic acid)	≤0.8	0.4 ± 0.18
Peroxide value (in milleq. O_2 per kg/oil)	≤20	7.0 ± 3.8
K_{232}	≤2.50	1.35 ± 0.18
K_{270}	≤0.22	0.10 ± 0.02
ΔK	≤0.01	0.001 ± 0.0013

2.5. Phenolic Compounds

Identification and quantification of phenolic compounds was performed for each olive oil sample. Phenols were extracted according to IOC procedure (COI/T.20/Doc. No 29/Rev.1 2017) and identified according to Grilo et al. [3]. Specifically, in a centrifuge tube, 2 g of olive oil were mixed with 5 mL of a solution of methanol and water (80:20 v/v). The tube was vortexed for 1 min, held in an ultrasonic bath for 15 min, and centrifuged at 5000 rpm for 25 min. The supernatant was filtered with a 0.45 μm PTFE syringe filter and kept at 4 °C. Triplicate samples of olive oil were used for each cultivar, planting density, and canopy position. Phenolic compounds were identified by ultra-high performance liquid chromatography, heated electrospray coupled with high resolution mass spectrometry (UHPLC-HESI-MS) analysis using a quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UHPLC analysis was performed using a Dionex Ultimate 3000 System (Dionex Softron GmbH, Germering, Germany) equipped with an autosampler controlled by Chromeleon 7.2 Software (Thermo Fisher Scientific, Bremen, Germany). A UHPLC column (Phenomenex Luna C18(2) 50 × 1 mm, 2.5 μ) was used for separation of the selected compounds at 35 °C. The mobile phases used were 0.1% formic acid in water (v/v) (A) and methanol (B). The elution gradient program was: 0–5 min 10% B; 5–50 min linear increase to 99% B, 50–56 min 10% B coming back to the initial conditions until full stabilization. The column temperature was set at 30 °C and the injection volume at 1 μL. The flow rate was 50 μL min^{-1}. The total UHPLC-HESI-MS method runtime was 60 min. Detection was based on calculated exact mass and on retention time of target compounds, and data were evaluated by Quan/Qual browser Xcalibur 3.0 (Thermo Fisher Scientific, San Jose, CA, USA). Reference phenolic compounds including hydroxytyrosol, tyrosol, oleocanthal, were purchased from Sigma-Aldrich (Steinheim, Germany). Linearity of the MS response was verified with solutions containing all standards at six different concentration levels over the range from 0.250 to 5 ppm. Each point of the calibration curve corresponded to the average of five independent injections.

2.6. Fatty Acid Profile

Fatty acids of oil samples were determined as methyl esters by gas chromatography using the method described by the International Olive Oil Council (IOOC/T20 doc. 33). Quantification was carried out using a Focus GC equipped with a MEGA 10 column (50 m × 0.32 mm × 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) and helium as carrier gas. Data were expressed as percentage of total area of the picks from each chromatogram.

2.7. Sensory Evaluation

Sensory evaluation of the oils was performed according to the panel test method (IOOC/T20 Doc. 15/Rev. 10) by trained panelists from the Assessorato Regionale dell'Agricoltura, dello Sviluppo Rurale e della Pesca Mediterranea (Sciacca, Italy). This method is only applicable to olive oils and is based on the intensity of attributes perceived by a group of tasters trained and monitored as a panel. The main positive attributes are fruity, bitter, and pungent. Four

samples were evaluated in each session. Samples were evaluated in individual sensory booths, using standard blue glasses. Judges could re-taste the oils as often as necessary and had to rinse their mouth with water and eat crackers in between sample tastings. Each session lasted approximately 60 min. Oils were tasted two times on different days and results are expressed in median of the sensory attribute.

2.8. Volatile Profile

Volatile compounds were analyzed using a gas chromatograph (GC) coupled with a mass spectrometer (MS) according to Sacchi et al. [31]. Each sample (3.0 ± 0.1 g), spiked with 4-methyl-2-pentanol as internal standard (2 mg/kg), was weighed into a 10 mL glass vial, and sealed with a PTFE/silicon septum. After 10 min at 40 °C, a solid-phase microextraction (SPME) fiber (DVB/CAR/PDMS, Sigma-Aldrich, St. Louis, MO, USA) was exposed to the sample headspace for 30 min for volatile extraction. The volatile analysis was performed on a GC/MS Shimadzu model QP5050A (Kyoto, Japan). A Supelcowax 10 (60 m × 0.32 mm × 0.5 µm, Sigma-Aldrich, St. Louis, MO, USA) was used for separation of compounds. After sampling, the fiber was thermally desorbed in the GC injector for 10 min at 240 °C. Helium was used as carrier gas at a flow rate of 1 mL/min. GC oven temperature started at 40 °C and ramped at 3.5 °C/min after 4 min to a final temperature of 240 °C. Ionization energy of 70 eV was adopted and the ions were analyzed in the m/z range from 40 to 400. Quantification was determined in relation to the internal standard. Peak areas were calculated by using the Labsolution acquisition system (GC-MS Solution version 1.20; Shimadzu, Kyoto, Japan). A blank test was performed prior to each analysis and all analyses were performed in triplicate. C5 (1-penten-3-one and 1-penten-3-ol) and C6 (Hexanal, *(E)*-2-hexenal, 3-hexen-1-ol acetate, 1-hexanol, 3-hexen-1-ol and 2-hexen-1-ol) volatile compounds were identified.

2.9. Statistical Analysis

Analysis of variance (ANOVA) at $p \leq 0.05$ was performed to identify the effects of cultivar and planting density on yield parameters (two-factor ANOVA), and the effects of cultivar, planting density, and canopy position on all quality parameters (three-factor ANOVA) using Systat software (Chicago, IL, USA). When appropriate, ANOVA was followed by Tukey's multiple comparison test ($\alpha \leq 0.05$) to separate means.

3. Results and Discussion

3.1. Production and Fruit Quality

The influence of cultivar, planting density, and canopy position on production and fruit characteristics is presented in Table 1. Fruit yield ranged between 4.9 and 9.2 kg/tree. MD trees of both cultivars showed higher fruit yield than HD trees. The same trend was observed for yield efficiency with MD giving the highest values (0.2 kg cm^{-2}). The highest yield per hectare was obtained with 'Cerasuola' at HD. These results are in agreement with a long-term study on 'Arbequina', where fruit and oil production per tree responded markedly to row spacing after six years from planting with higher values at a greater planting distance [32]. In 'Cerasuola', fruit yield expressed in t ha^{-1} indicated that lower fruit yield per tree of HD was compensated by the larger number of trees per hectare. In contrast, 'Koroneiki' did not show significant differences between HD and MD, suggesting that yield potential of a single 'Koroneiki' tree was reached at lower densities. Results on the percentage of fruit yield harvested in the upper or lower layers of the canopy demonstrated that the majority of the production occurred in the upper layers. 'Cerasuola' presented the biggest differences in fruit distribution across the canopy. Percentage of fruit production in the lower layers of the canopy was higher than previously described by Castillo-Ruiz et al. [33] for 'Arbequina' in the south of Spain (17%), indicating that cultivars (growth habit, canopy density, and vigor) and cultural practices (mainly pruning) have a great influence on fruit distribution within the canopy.

'Cerasuola' presented higher fruit weight than 'Koroneiki' (Table 2). Fruit weight was not affected by planting density or canopy position, suggesting that any possible difference in fruit growth must have been canceled by the fruit's ability to attract assimilates from distant areas of the canopy during development [34].

Table 2. Production and fruit quality of 'Cerasuola' and 'Koroneiki' olive trees planted at 1000 (HD) and 500 (MD) trees/ha. Production described as fruit yield (kg/tree), yield efficiency (kg cm^{-2}), yield (t ha^{-1}), percentage of total fruit harvest distributed on the upper and lower layers of the canopy, and oil yield (kg of oil 100 kg^{-1} of fruit). Fruit conditions at harvest described by maturity index, percentage of black peel cover (veraison), fresh weight (g), moisture and fat content (g 100 g^{-1} of fresh weight).

Cultivar	**'Cerasuola'**				**'Koroneiki'**			
Planting Density	**HD**		**MD**		**HD**		**MD**	
Canopy Layer	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**
Fruit yield	6.82 ab [z]		8.03 a		4.40 b		9.23 a	
Yield efficiency	0.23 ab		0.26 a		0.16 b		0.28 a	
Yield	6.82 a		4.02 b		4.40 b		4.62 b	
% fruit production	60.7 a	39.3 c	61.6 a	38.4 c	58.4 ab	41.6 bc	52.5 abc	47.5 abc
Fruit weight	1.35 a	1.35 a	1.35 a	1.38 a	0.87 b	0.82 b	0.77 b	0.79 b
Maturity index	2.56 c	2.15 d	3.06 ab	2.79 bc	3.18 a	2.69 bc	2.68 bc	2.54 cd
% black peel	87.3 ab	62.7 c	95.8 a	79.6 ab	87.7 ab	72.6 bc	65.9 bc	71.6 bc
Oil yield [y]	15.7	15.4	18.5	14.9	21.2	15.6	17.3	13.1
Moisture content	49.5 c	53.7 a	51.4 bc	52.5 ab	49.5 c	50.5 bc	49.9 c	51.7 abc
Fat content	27.4 a	24.8 d	27.1 a	25.5 cd	27.6 a	25.8 bcd	26.9 ab	26.4 abc

[z] Values followed by the same letters in each row are not significantly different among cultivars and planting densities for the yield parameters, and among cultivars, planting densities and canopy position for fruit quality parameters by Tukey's test at $\alpha < 0.05$ (n = 20). [y] Only a single oil extraction per treatment combination, no statistical analysis.

During maturation, most of the chlorophyll is degraded and replaced by anthocyanins, producing a change in color from green to black in the olive fruit, commonly visually evaluated by the maturity index (MI). MI ranged between 2.2 and 3.3 and differences between upper and lower layers of the canopy were larger in HD than in MD (Table 2). This is likely due to a less homogeneous distribution of the light intercepted by the canopy when the trees are close to one another [35].

Percentage of black peel was assessed by image analysis. The same method applied in this study was previously used to study peel color and maturity level of oranges, apples, peaches, and nectarines at harvest [28,36,37]. In olive, percentages of black peel did not follow the same trend as MI values. In 'Cerasuola', MI of fruits from upper canopy layers of HD trees was significantly lower than fruits from MD trees, while their percentage of black peel was not significantly different. Differences between these methods can be explained by the color changes in the peel of the fruit from both cultivars and the methodology applied. In 'Cerasuola' fruits, the darkening of the peel starts on one side of the fruit and then spreads to the whole fruit, creating higher variability in the percentage of black peel. While in 'Koroneiki', the black peel starts from the tip of the fruit and uniformly expands around the fruit. To determine MI, fruits were separated into groups based on the amount of dark skin color, and in fruits where the color was more uniform, like 'Koroneiki', the MI relates better to the percentage of black peel. On the other hand, in 'Cerasuola', the MI methodology allows for determination of the intermediate color break between green and dark, detecting a greater difference between samples than the percentage of black peel. These results show how subjective the MI can be and how limiting the use of a nonsubjective method is at higher maturity stages. Furthermore, to decide the harvest date, many producers use MI in the field, but then the industry uses optical sensors to carry out a preselection of the fruits before oil extraction. Therefore, the results also showed how important the calibration of these sensors is to decrease the possible discrepancies between MI done in the field and the quality of the oil extracted.

Olives from the upper layers of the canopy produced more oil on average, regardless of cultivar and planting density (Table 2). However, fruit fat content did not follow the same pattern as oil yield, suggesting that other factors (i.e., fruit characteristics) can influence the oil yield even when the same extraction conditions are used [14,38]. Planting density and canopy position did not affect fruit moisture content of 'Koroneiki'. In contrast, 'Cerasuola' fruit showed higher moisture content in the lower than the upper canopy layers, but only at HD.

'Cerasuola' fruit showed higher fat content at the upper than the lower canopy layers independent of planting density, whereas 'Koroneiki' fruit showed higher values in the upper layers only at HD. These observations agree with findings from previous studies showing that fruits from lower canopy positions accounted for approximately one-fourth of tree fruit production (26%) and oil yield (25.2%) [33], while fruits from the upper canopy layers showed the highest weight, ripening index, and oil content [12,26,32]. A possible explanation for a higher oil accumulation is that fruits from upper layers were more exposed to incident light which has been shown to increase the number of oil bodies inside the fruit mesocarp [39].

3.2. Oil Quality and Minor Compound Composition

Chlorophylls are mostly located in the skin of the olive fruit, where the highest photosynthetic activity is observed. Due to their liposolubility, chlorophylls migrate to the oil phase during the extraction process [40]. The final concentration in the oil is affected by the initial concentration in the fruit, but also by extraction variables [14,41]. Cultivar and planting density showed no effect on chlorophyll and carotenoid contents in the oil (Table 3). However, these pigments were significantly higher in oils from the lower than the upper layers.

Table 3. Oil chlorophyll, carotenoid content, ratio of unsaturated and saturated fatty acids (UFA/SFA), ratio of monounsaturated and polyunsaturated fatty acids (MUFA/PUFA), and main phenols in 'Cerasuola' and 'Koroneiki' olive trees planted at 1000 (HD) and 500 (MD) trees/ha. Chlorophyll, carotenoids, 3,4-DHPEA-EA, 3,4-DHPEA-EDA, *p*-HPEA-EDA, *p*-HPEA-EA, hydroxytyrosol, tyrosol, sum of total phenols, sum of C5 volatiles, and sum of C6 volatiles were determined in olives from the upper and lower layers of the canopy and expressed as mg kg^{-1}.

Cultivar	**'Cerasuola'**				**'Koroneiki'**			
Planting Density	**HD**		**MD**		**HD**		**MD**	
Canopy Layer	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**
Chlorophyll	13.1 b *	17.3 a	9.52 b	20.0 a	10.2 b	14.1 a	13.0 b	18.0 a
Carotenoids	10.2 b	12.5 a	8.03 b	14.2 a	7.75 b	10.7 a	9.89 b	13.2 a
UFA/SFA	5.67 b	6.26 a	4.70 d	6.15 a	4.97 cd	5.20 c	5.21 c	5.68 b
MUFA/PUFA	5.40 d	5.45 d	5.18 d	5.63 d	8.05 ab	7.11 bc	8.35 a	6.73 c
3,4 DHPEA-EA	368 b	187 d	248 c	169 d	434 a	383 b	462 a	185 d
3,4 DHPEA-EDA	75.1 a	70.3 b	66.9 b	59.0 c	21.6 e	20.8 e	25.9 d	12.3 f
p-HPEA-EDA	23.8	23.8	23.5	23.6	23.8	23.7	23.7	23.7
p-HPEA-EA	116 d	137 bc	127 cd	112 d	165 a	117 d	148 b	87.4 e
Hydroxytyrosol	9.68 e	9.80 e	15.0 d	7.46 f	6.69 g	21.7 b	16.1 c	29.2 a
Tyrosol	30.5 b	25.2 bc	22.3 c	25.8 c	27.0 bc	39.9 a	38.2 a	30.5 b
Σ phenols	734 c	564 e	626 d	507 f	776 b	705 c	816 a	467 g
Σ C5	0.62 a	0.22 b	0.59 a	0.74 a	0.34 b	0.25 b	0.32 b	0.10 b
Σ C6	21.2 b	13.5 bc	43.1 a	12.8 bc	10.4 bc	7.86bc	10.7 bc	3.13 c

* Values followed by the same letters in each row are not significantly different by Tukey's test at $\alpha < 0.05$ ($n = 3$).

Identified fatty acids were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1n9), margaric (C17:0), stearic (C18:0), oleic (C18:1n9), linoleic (C18:2n6), linolenic (C18:3n3), arachidic (C20:0), gadoleic (C20:1n9), behenic (C22:0), and lignoceric (C24:0) acids. In order to facilitate the interpretation of fatty acid compositional changes in response to the studied factors, fatty acids were grouped according to their saturation level into saturated fatty acids (SFA; myristic, palmitic, margaric, stearic, arachidic, behenic, and lignoceric acids)

and unsaturated fatty acids (UFA; palmitoleic, oleic, linoleic, linolenic, and gadoleic); and further into monounsaturated fatty acids (MUFA; palmitoleic, oleic, and gadoleic) and polyunsaturated fatty acids (PUFA; linoleic and linolenic). MUFAs are the predominant fatty acids in olive oil, with oleic acid being the most abundant, ranging from 55% to 83% of total fatty acids [42]. The monounsaturated profile of fatty acids is one of the factors that may explain the healthy benefits of olive oil in the Mediterranean Diet [43,44]. In all of the measured samples, oleic acid was always the predominant fatty acid (70.03–72.47%). Cultivar showed a main effect on fatty acid ratios, with 'Cerasuola' showing a significantly higher UFA/SFA but a lower MUFA/PUFA than 'Koroneiki'. Canopy position also affected fatty acid ratios, with oils from lower layers showing significantly higher UFA/SFA. In 'Koroneiki', higher MUFA/PUFA in the oils from upper layers than in lower layers was due to a decrease of linoleic acid rather than changes of oleic acid. In contrast, an increase of linoleic acid in the oils from upper canopy layers was previously reported in 'Arbequina' and 'Frantoio' and justified by more mature olives [12,45]. There is no easy explanation for this discrepancy between our results and the previous literature, but a combination of greater light interception and lower crop load of the upper canopy layers in 'Koroneiki' may be in part related to the observed MUFA/PUFA trends.

Oleuropein and ligstroside are the most relevant phenolic compounds identified in olive fruit [46]. These substances are hydrolyzed after crushing by the enzyme β-glucosidase, leading to the formation of oleacein and oleuropein aglycon (respectively, 3,4-DHPEA-EDA and 3,4-DHPEA-EA) and oleocanthal and ligstroside aglycon (respectively *p*-HPEA-EDA and *p*-HPEA-EA), which exhibit a higher lipophilicity and constitute the most abundant phenolic compounds in virgin olive oil [47]. In addition to β-glucosidase, polyphenol oxidase and peroxidase degrade phenolic compounds during the extraction, also shaping the phenolic profile of the oil [48]. Cultivar significantly affected the phenolic composition of the oils (Table 3). 'Cerasuola' presented significantly lower phenol contents compared to 'Koroneiki', except for 3,4-DHPEA-EDA, which was higher in the former cultivar. There was no difference in *p*-HPEA-EDA level between cultivars. The phenol content was significantly higher in oils from HD than MD trees, except for hydroxytyrosol that showed higher values in oils from MD trees. Tyrosol was not affected by planting density. Canopy position significantly affected the phenol content in the oil. Oils from fruits in upper layers showed the highest total phenol content due to higher 3,4-DHPEA-EA, 3,4-DHPEA-EDA, and *p*-HPEA-EA, but lower hydroxytyrosol. Tyrosol and *p*-HPEA-EDA were not affected by canopy position. These results are in accordance with previous studies on 'Arbequina' and 'Frantoio', where canopy position was a determining factor for phenol concentration, increasing with height [12,45].

Volatile compounds are responsible of the fruity and green aroma of fresh olive oil [49]. These compounds are synthesized during processing from free polyunsaturated fatty acids, through an enzymatic pathway involving two main enzymes, lipoxygenase and hydroperoxide lyase. Lipoxygenase catalyzes the oxygenation of polyunsaturated fatty acids (linoleic and linolenic) to produce their corresponding 13-hydroperoxides. Hydroperoxide lyase catalyzes the cleavage of linoleyl and linolenyl 13-hydroperoxides, yielding C5 and C6 aldehydes, the main compounds identified in olive oil [50]. C6 volatiles presented the highest concentration in all of the samples, with *(E)*-2-hexenal being the major volatile compound. *(E)*-2-Hexenal concentration ranged between 13.73 and 0.89 mg kg^{-1} for 'Cerasuola' and between 3.58 and 0.47 mg kg^{-1} for 'Koroneiki', in oils from HD and MD trees, respectively. In this study, C5 and C6 volatile compounds were affected by cultivar and canopy position. Both families of volatile compounds were highest in oils from 'Cerasuola' and upper layers of the canopy (Table 3). Fruit from upper layers of the canopy of 'Arbequina' were previously reported to have higher volatile compounds concentration than fruit from lower layers after November and a MI higher than 1 [12].

3.3. Oil Sensory Attributes

From a sensory point of view, all the samples can be classified as extra virgin olive oil with medium intensity of the positive attributes. The artichoke (*Cynara cardunculus*), almond (*Prunus dulcis*), and tomato attributes, very common in Sicilian olive oils, were also found in the extracted oils. 'Cerasuola' oils were characterized by higher artichoke, almond, tomato, and oregano (*Origanum vulgare*) attributes than 'Koroneiki' oils (Table 4). On the other hand, 'Koroneiki' oils were distinguished by low fruity and banana (*Musa* spp.) attributes. Bitter and pungent sensations were previously positively related to the amount of secoiridoid compounds [51]. In particular, *p*-HPEA-EDA is known to be responsible for pungency in the oil [51]. In this study, values of *p*-HPEA-EDA were not affected by cultivar, planting density, or canopy position although a slight increase of pungency was found in oils from upper canopy layers and HD. C6 volatiles were previously associated with bitterness and pungency in olive oil [52]. In our study, from the upper layer of the canopy, the highest C6 volatile concentration was followed by an increase in pungency in the oils. Moreover, most C5 volatile compounds in addition to the C6 volatiles contribute to almond (mainly *(E)*-2-hexenal), tomato (mainly *(E)*-2-hexen-1-ol), and banana (mainly *(Z)*-3-hexenyl acetate) odor notes [53]. The contribution of volatile compounds to the overall aroma of virgin olive oil depends not only on their concentration but also on their sensory threshold values [54,55]. Consequently, a high concentration of volatile compounds is not necessarily related to a major contribution to the oil aroma [54,56].

Table 4. Quantitative descriptive profile for sensory evaluation of 'Cerasuola' and 'Koroneiki' oils obtained from two planting densities and two fruit canopy positions. Numbers represent the median of the attributes (n = 8) according to the international olive oil council (COI/T.20/DOC.15/Rev.10) standards.

Cultivar	**'Cerasuola'**				**'Koroneiki'**			
Planting Density	**HD**		**MD**		**HD**		**MD**	
Canopy Layer	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**	**Upper**	**Lower**
Fruity	5	6	5	5.5	3	3	3	2.8
Bitter	5	6	5.5	5	5	5	5	4
Pungent	6	5.5	6	5	5	4	4	3
Artichoke	3	3.5	3	3.5	2	nd [z]	2	nd
Almond	2	2	2.5	nd	1	nd	1	nd
Grass	2.5	3	nd	3	nd	nd	nd	nd
Green tomato	2.5	3	nd	3.5	nd	1	nd	nd
Banana	nd	nd	nd	nd	2	nd	1	1
Oregano	nd	1	nd	2	nd	nd	nd	nd
Chicory	nd	1	1	1	nd	nd	nd	nd

[z] nd = not detected.

4. Conclusions

Given the relevance of harvest costs to the overall cost of olive oil production, tree density in newly planted olive orchards has been increasing steadily as part of the intensification of olive tree cultivation. Data obtained in this study showed that higher planting density increased yield per hectare of the Sicilian cultivar 'Cerasuola', which was proven to adapt to higher density orchards, without losing the quality and peculiar sensory attributes. Upper layers of the canopy were characterized by higher crop loads and more mature fruits with higher fat content. All the major variables had an influence on fatty acid, phenol, and volatile composition. Canopy position was the primary factor that influenced most of the measured parameters. The differences in production, fruit maturity, and fat content between upper and lower canopy layers increased at higher densities. On the other hand, treatment effects on oil quality did not follow the same trend in both cultivars, showing that genetic factors interacted with environmental conditions to influence oil quality. Taken together, the impact of the interactions between cultivar, planting density, and canopy position on oil quality and sensory properties established in this study provide new insights

into the relationships between yield and tree structure in modern high and medium density hedgerow olive planting systems.

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Is There Daily Growth Hysteresis versus Vapor Pressure Deficit in Cherry Fruit?

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Abstract: The growth of cherry fruit is generally described using a double sigmoid model, divided into four growth stages. Abiotic factors are considered to be significant components in modifying fruit growth, and among these, the vapor pressure deficit (VPD) is deemed the most effective. In this study, we investigated sweet cherry fruit growth through the continuous, hourly monitoring of fruit transversal diameter over two consecutive years (2019 and 2020), from the beginning of the third stage to maturation (forth stage). Extensometers were used in the field and VPD was calculated from weather data. The fruit growth pattern up to the end of the third stage demonstrated three critical steps during non-rainy days: shrinkage, stabilization and expansion. In the third stage of fruit growth, a partial clockwise hysteresis curve of circadian growth, as a response to VPD, appeared on random days. The pattern of fruit growth during rainy days was not distinctive, but the amount and duration of rain caused a consequent decrease in the VPD and indirectly boosted fruit growth. At the beginning of the fourth stage, the circadian growth changed and the daily transversal diameter vs VPD formed fully clockwise hysteresis curves for most of this stage. Our findings indicate that hysteresis can be employed to evaluate the initial phenological phase of fruit maturation, as a fully clockwise hysteresis curve was observable only in the fourth stage of fruit growth. There are additional opportunities for its use in the management of fruit production, such as in precision fruit farming.

Keywords: sweet cherry; fruit growth; hysteresis; fruit maturation; vapor pressure deficit (VPD)

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1. Introduction

Sweet cherry (*Prunus avium* L.) is a non-climacteric fruit. Most cherry cultivars have red skin with colored flesh at maturation, although some cultivars manifest yellow-red skin with cream-colored flesh and colorless juice, thereby extending their coloration from red to purple and cream to pink [1]. The optimal time for evaluating cherry fruit quality characteristics is determined by changes in skin color and the accumulation of glucose and fructose, which coincide with a fast increase in fruit size [2]. In the maturation period, increasing the fruit weight and anthocyanin content follow sigmoid patterns, whereas the lightness (L), redness (a) and yellowness (b) experience a significant linear decrease during the maturation period [2,3]. Sweet cherry fruit growth is described by a double-sigmoidal pattern, divided into four growth stages. During stage one, cell division in the pericarp occurs. In stage two, the weight remains constant and the endocarp hardens through the lignification of its tissue, while the embryo develops. Stage three is characterized by the rapid expansion of the fruit mesocarp and by an increase in weight [4], whereas stage four begins with fruit maturation and finishes with fruit drop. According to research by Gucci et al. [5], Corelli-Grappadelli and Lakso, [6] and Hammami et al. [7], the fruit maturation process is influenced and regulated by endogenous (biotic) factors, such as

genetic differences and fruit load, and exogenous (abiotic) factors, such as water availability and ambient temperature.

Sweet cherry is highly perishable and is greatly affected by orchard management and environmental conditions [8]. Precision farming is a recent management approach which has been applied in order to increase farming output with less input, through the use of digital technology. A main research focus in precision farming consists in monitoring fruit growth as a continuous recording of fruit diameter with sensors. The observation of circadian cycles, applied to fruit growth, has contributed to the gathering of information regarding this phenological stage and has yielded data for the development of precision farming technologies. Monitoring fruit diameter has been carried out in various studies by researchers measuring diurnal changes in the volume of selected fruits, including peaches [9], grapes [10], kiwifruit [11], sweet [12,13] and pears (Morandi et al., 2014) [14]. Cherry fruit growth during stage 2 and early stage 3 of fruit growth revealed diurnal patterns characterized by net losses of fruit volume during the day and net gains during the night; however, during late stage 3 the diurnal pattern disappeared [12]. In addition to this, fruit growth is highly sensitive to water deficits, which decreases the fruit's biomass accumulation and tissue expansion, while reducing cell numbers [15]. According to Manfrini et al. [16], seasonal fruit monitoring gives researchers the opportunity to forecast the fruit load per tree, fruit size and harvesting time, while also leading operators to adopt more accurate thinning and irrigation practices. Regulated deficit irrigation (RDI) is another benefit which can be extracted from fruit growth monitoring and which has been applied in precision agriculture management for the optimization of irrigation water, without a reduction of yield or fruit quality. A common challenge associated with tree-based sensors is the adjustment of their output to physiologically meaningful parameters in a consistent manner [17,18]. The phenomenon of hysteresis has been known for a long time, attracting the attention of many investigators for years. The reason is that hysteresis is ubiquitous [19]. In Ancient Greek, hysteresis means "to lag behind". Brady and Weil [20] defined it as: "A relationship between two variables that changes depending on the sequences or starting point. An example is the relationship between soil water content and water potential, for which different curves describe the relationship when a soil is gaining water or losing it". In other words, when the time argument of an input function is stretched or compressed, the corresponding output function is not stretched in the same way [21,22].

In cherry plants, Oyarzún et al. [23] observed a hysteresis trend in the diurnal path of transpiration vs. leaf water potential, which roughly described a figure-eight pattern. A similar pattern was observed in laboratory studies by Brüggenwirth and Knoche [24] when they investigated the cracking susceptibility of the fruit. They measured the total strain of mature cherry exocarp affected by the pressure applied by an elastometer to simulate the internal pressure of fruit during maturation. This pressure is connected with fruit volume variations and depends on plant water flow and hence on the vapor pressure deficit (VPD). Therefore, we hypothesize that VPD could influence fruit growth, along with hysteresis behavior, in the field.

In this study, we examined daily changes in transversal diameter versus VPD in cherries to verify the presence of differing hysteresis curves in different phenological stages of fruits. The objective of this work was to describe the sweet cherry growth by means of an automatic extensometer within a 2-year time frame (2019 and 2020), for about 33 days, from the beginning of the third stage of fruit growth to fruit maturation. The need for this study was justified by the fact that no previous studies had been conducted on sweet cherries to evaluate the hysteresis cycle vs VPD in terms of of the diurnal variation of fruit growth.

2. Materials and Methods

2.1. Site Description and Phenology

This study was conducted in the experimental research station and botanical garden of Polytechnic University of Marche, at Gallignano, in Ancona (AN), Italy (43°34′06.2″ N 13°25′24.2″ E). The station's 20-year-old sweet cherry orchard, cultivar Blaze Star, grafted on MAXMA 14, was used. The trees were planted in 4.5 m × 3.0 m and trained as a vase system, under organic agriculture management, in rainfed conditions and deep silt clay soil. According to the Köppen–Geiger climate classification, Gallignano belongs to the Cfb category, characterized by warm temperatures and a fully humid and warm summer [25]. The flowering in 2019 was from 29 March 29 to 16 April 16, with the peak on 8 April 8; in 2020, it was from 3 April to 20 April, with the peak on 11 April.

2.2. Fruit Growth

2.2.1. Automatic Extensometer

Fruit diameter was measured in both years from the beginning of third stage of fruit growth to fruit maturation, according to weather conditions. In 2019 the growth of fruit 3 was measured from 4 May (26 DAFB) to 4 June (day of the year—DOY 124 to DOY 155). Fruit 1 growth was measured from 4 May (DOY 124) but dropped on 10 May (DOY 130) and it was substituted with another fruit (fruit 2), which was actively growing until 4 June (DOY 155). In 2020, the growth of one cherry fruit (fruit 4) was measured from 23 April (12 DAFB) to 25 May (DOY 114 to DOY 146).

An electronic extensometer (DEX20, manufactured by Dynamax Inc., Huston, TX, USA), capable of measuring the growth of small fruits (0–25 mm), was used. The DEX20 extensometer is a caliper style device with a full-bridge strain gage attached to a flexible arm and with an output signal range of ±5 mV. The millivolt sensor output measured both the diurnal and long-term growth of fruit. Data were recorded using a Campbell scientific CR100X unit at 1-h intervals and sent to our cloud service, based on Amazon Web Services (AWS), twice per day (Figure 1). The measured daily data were normalized using the Min-Max method with the following equation.

$$x' = 0.9*((x - x\text{min})/x\text{max} - x\text{min})) + 0.05 \quad (1)$$

where x' is the normalized value, x is the value of the existing data, and $x$min and $x$max are the minimum and maximum values of the data, respectively.

Figure 1. Acquisition system, with a CR100X datalogger (Campbell Scientific Inc., Logan, UT, USA) and an automatic extensometer DEX20 (Dynamax Inc., Huston, TX, USA).

Cherry fruit growth was divided in two time periods according to fruit growth stage. The first period started around the beginning of the third fruit growth stage and finished at the end of this growth stage from DOY 131 to 150 in 2019, and from DOY 114 to 141 in 2020. The second period included the fourth fruit growth stage (i.e., the maturation stage) for a duration of 5 days in both years.

2.2.2. Manual Electronic Caliper

Manual measurements of fruit diameter were performed to represent growth phases in the final part of the double sigmoid curve as a reference for automatic extensometer measurements. One branch per tree from 10 homogeneous trees was randomly selected and on each branch 5 cherry fruits were tagged. The transversal diameter of the tagged fruits was measured from the beginning until the end of the experiment, every 3 to 5 days, with a manual electronic caliper. In the event of fruit abscission, a fruit of comparable size and on the same branch was selected.

2.3. Meteorological Data

Weather data were collected from a Vantage pro2 precision weather station (Davis Instruments Corporation, Hayward, CA, USA) located 500 m away from the cherry orchard. The weather data were automatically transferred to a computer using Vantage pro2, which consisted of weather link software and a data logger. Vapor pressure deficit (VPD) was calculated based on relative humidity (RH) and air temperature (T °C) data, collected from the weather station, with formulas recommended by Monteith and Unsworth [26].

$$\text{VPD} = (1 - (\text{RH}/100))*\text{SVP and SVP (Pascals)} = 610.7*10^{7.5\text{T}/(237.3+\text{T})} \quad (2)$$

where SVP is saturated vapor pressure.

According to the weather station, the sunrise time for all days of the experiment in 2019 and 2020 was set at 6 AM. Our instrument was set to legal Rome time.

2.4. Hysteresis Curve

Hysteresis is non-linear loop-like behavior that does not show affine similarity with respect to time [21,27]. For the description of hysteresis curves, clockwise and anticlockwise loops are referred to. Additionally, the determination of the proper time period for realizing whole-day pictures, as well as seasonal patterns of hysteresis curves, is necessary. Bai et al. [28] suggest dividing daytime hours, as well as growing season, into different periods. Therefore, with consideration of the fruit growth trend over 24 h and the response to VPD, as well as the growth stage of the fruit, the experiments in both years were divided into two periods. In 2019, the first period was from 11 May to 30 May (DOY 131 to 150) and the second period was from 31 May to the end of the experiment, on 4 June (DOY 151 to 155). In 2020, the first period was 23 April to 20 May (DOY 114 to 141) and the second period was from 21 May to 25 May (DOY 142 to 146). Moreover, the graphical representation of daily fruit growth and its fluctuations was reported from 6 AM (approximately the time of sunrise) until 5 AM the next day.

2.5. Data Analysis and Presentation

Data were analyzed using correlation and regression analysis (JMP®, Version 8.0.2.2 SAS Institute Inc., Cary, NC, USA, 1989–2019) and the graphs were drawn using SigmaPlot 11.2 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Fruit Growth Monitoring

The growth season monitored in 2019 was 9 days longer than that in 2020 (Figure 2). Accordingly, the hourly VPD mean for 32 and 33 days was 0.37 ± 0.39 kPa in 2019 and 0.633 ± 0.48 kPa in 2020, respectively.

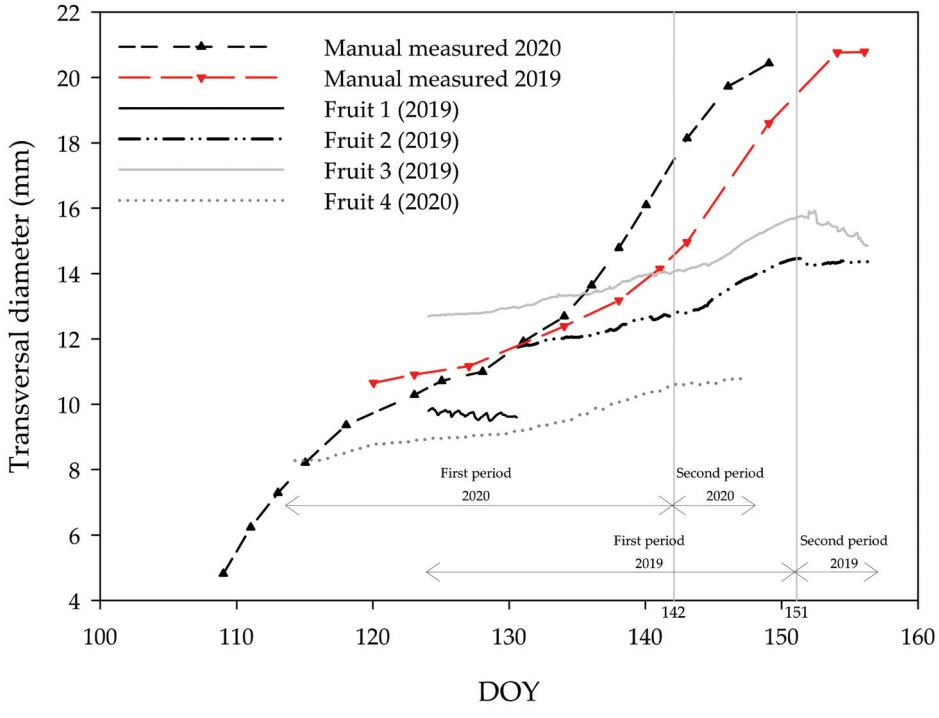

Figure 2. Data interpolation of the 50 labeled fruit in 2019 (manually measured 2019) and 2020 (manually measured 2020); fruit 1 is related to extensometer 1 in 2019; fruit 2 is related to extensimeter 1 in 2019; fruit 3 is related to extensometer 2 in 2019; fruit 4 is related to extensometer 1 in 2020. The first period of the study was from DOY 131 to 150 in 2019 and from DOY 114 to 141 in 2020. The second period of the study was from DOY 151 to 155 in 2019 and from DOY 142 to 146 in 2020.

Comparing manual and automatic diameter measurements, in the first period (third fruit growth stage) of both years of the experiment, the hourly measured transversal diameter by the extensometer showed a similar pattern of growth in all fruits, and the growth was comparable with the trend observed with manual caliper measurements (Figure 2). However, in the second period in both years, the trends of the transversal diameter of the fruits measured using the extensometer were different from those that were manually measured. It could be hypothesized that with the softening of the mesocarp [29], the pressure of extensometer arms acted as a suppressing force. This suppressing force caused the non-similarity of the trends measured using the manual calipers in comparison with those measured using the automatic extensometer. However, the double sigmoidal pattern of growth was maintained in both measuring methods. In addition, the extensometer monitored hourly changes in fruit diameter and the data were reported on the same graph, whereas the manual measurements were interpolated in a continuous line.

In the third fruit growth stage, the most frequent daily pattern of the hourly-measured transversal diameter can be described by three phases (Figure 3) and is comparable to a third-degree polynomial function (66 out of 82 days with a $R^2 \geq 0.75$).

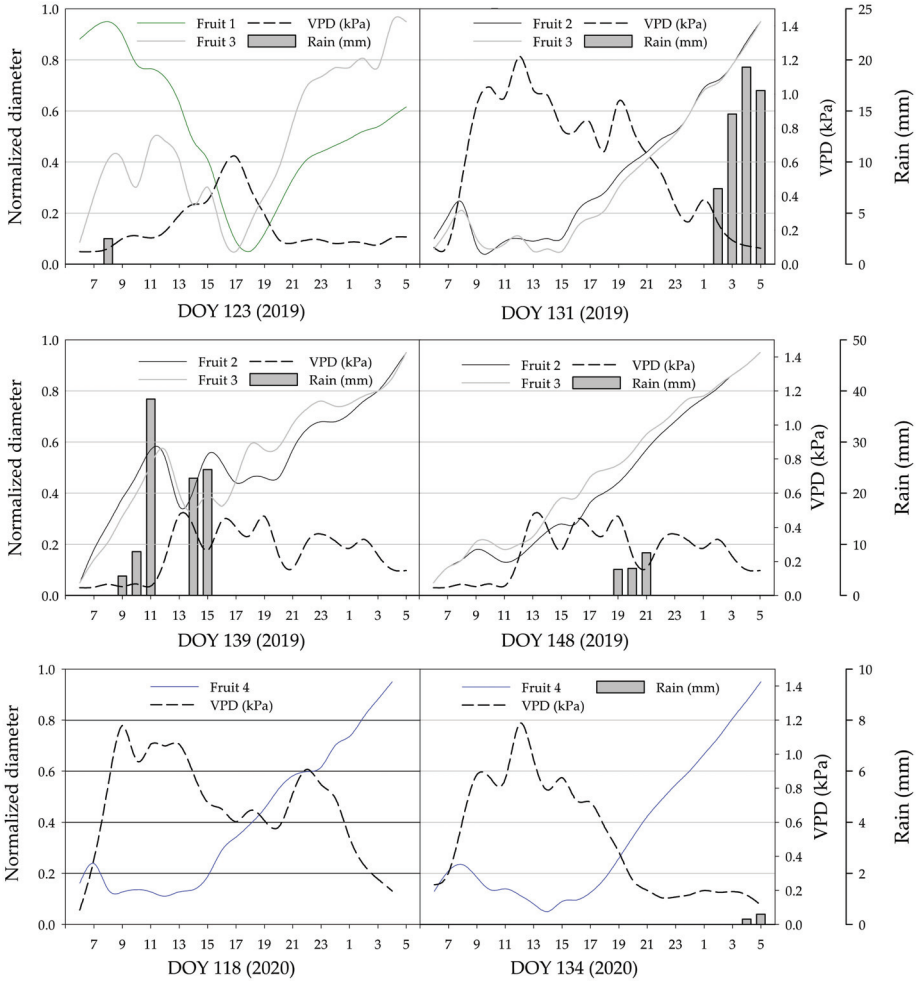

Figure 3. Most representative daily trends of transversal diameter, VPD and rain in the first periods (growth stage 3) in 2019 and 2020. The three-phase daily trend on DOY 118 in 2020 and DOY 131 in 2019; the v-trend of fruit 1 on DOY 123 in 2019; the rain influence on DOY 123, 131 and 139 in 2019 and DOY 141 in 2020.

The first daily phase started at approximately 8.25 a.m. (dev. St. ± 1.38 h) and lasted a few hours. During this phase, the transversal diameter decreased while the VPD increased. The second daily phase revealed substantial stability of the transversal diameter, which overlapped with the maximum VPD values. Then, the results observed in the third phase were opposite to those observed in the first daily phase. This trend started when VPD was at its maximum and lasted until the next day. Fruit diameter increased while VPD decreased.

This daily trend was observable in 58% of the cases in 2019 (14/25 days for fruit 2 and 19/32 days for fruit 3) and 61% in 2020 (20/33 days for fruit 4).

For fruit 1, we observed a decrease in the transversal diameter, followed by a similar increase, with a daily V-shaped trend (Figure 3) until the drop-down of the fruit, which was not able to reach maturity (DOY 130, Figure 2). The daily transversal diameter trend was the opposite of the VPD trend. For fruit 1, when the VPD increased approximately

from sunrise to sunset, negative linear regression versus transversal diameter was detected. Nevertheless, fruits 2, 3 and 4 did not exhibit any kind of regression.

The rain did influence the daily trend of the transversal diameter. When it rained after sunset or in the first hours after sunrise, no response was observed and the transversal diameter followed the three-daily phases (for instance, on DOY 131 of 2019, Figure 3). During the daily phase with increasing VPD, a positive response of transversal diameter to the rain was observed (for instance, DOY 123 and 139 of 2019, Figure 3). However, continuous transversal diameter growth was detected in the days with many hours of rain (for instance, DOY 141 of 2020, Figure 3).

In the second period of both years the fruits monitored using the extensometer did not show a distinct pattern in terms of a daily trend; moreover, the trends of fruits 2, 3 (2019, from DOY 151 to DOY 155) and 4 (2020 from DOY 142 to DOY 146) were different (Figure 2).

For fruit 2, the transversal diameter on DOY 152, 153 and 155 at the end of each day was larger than at the starting point of the same day. However, on DOY 151 and 154 the opposite phenomenon occurred. The diameter decreased by 0.7% during the 5 days of this period (from 14.467 mm to 14.372 mm, Figure 4).

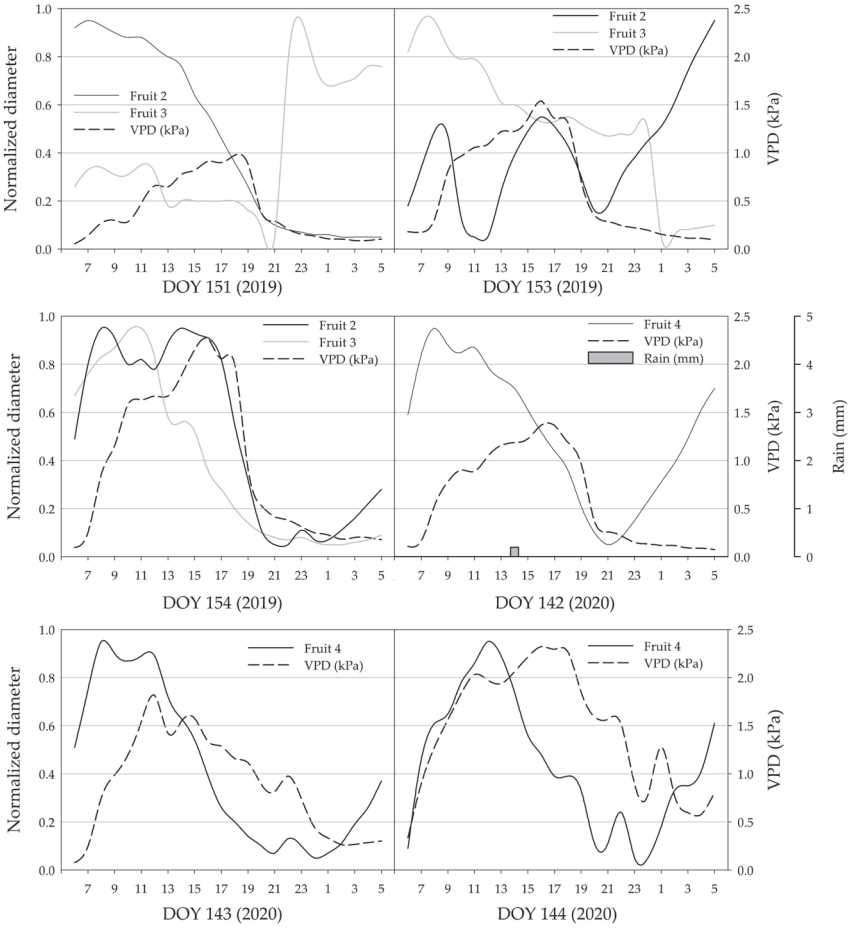

Figure 4. Most representative daily trends of transversal diameter, VPD and rain in the second periods (growth stage 4) in 2019 and 2020.

For fruit 3, on all days except DOY 151, the transversal diameter at the beginning of the day was larger than it was at the end of the same day, decreasing by 5.7% globally (from 15.748 to 14.855 mm, Figure 4).

For fruit 4, on all days except DOY 143, the transversal diameter at the end of the day was larger than at the beginning of the same day, rising by 1.05% (from 10.626 mm to 10.737 mm, Figure 4).

3.2. Analysis of Diameter Growth versus VPD and Hysteresis Curves

In the first periods in 2019 and 2020, the relationship between the VPD and transversal diameter of fruits 2, 3 and 4 did not follow a similar pattern every day. In fact, in some days the trend appeared chaotic, whereas in others it followed a partial clockwise loop (Figure 5, Tables 1–4). Since the observed loops were not representative of the whole day, they can be counted as a partial clockwise hysteresis curve.

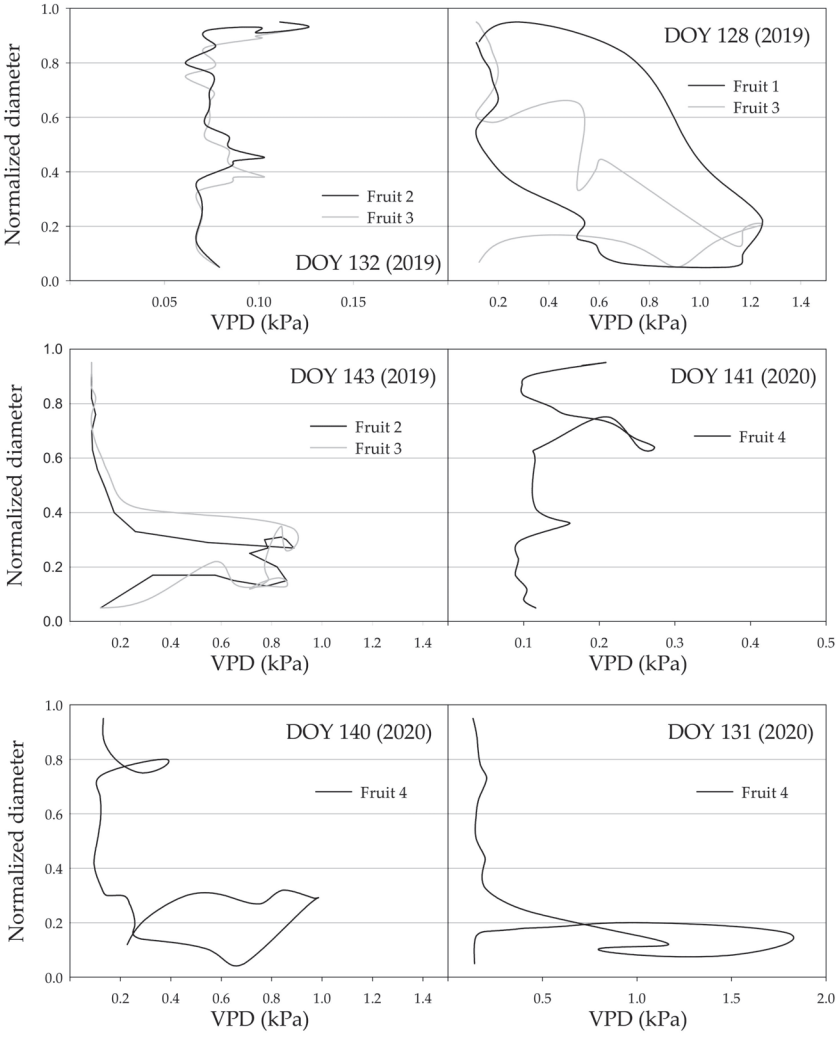

Figure 5. Most representative relations between VPD and transversal diameter in fruit growth stage 3 in 2019 and 2020.

Table 1. Data of daily mean VPD and rainfall for 2019.

DOY	Daily Mean of VPD (kPa)	Rain Fall	Hysteresis Curve		
			Fruit 1	Fruit 2	Fruit 3
124	0.22	Yes	Full		Partial
125	0.17	Yes	Full		Partial
126	0.24	No	Full		Partial
127	0.42	No	Full		No
128	0.47	Yes	Full		Partial
129	0.20	Yes	No		Partial
130	0.50	No			Partial
131	0.60	Yes		No	No
132	0.08	Yes		No	No
133	0.15	Yes		No	No
134	0.19	Yes		Partial	Partial
135	0.19	Yes		Partial	Partial
136	0.25	No		No	No
137	0.32	No		Partial	Partial
138	0.19	Yes		Partial	Partial
139	0.25	Yes		No	No
140	0.50	No		Partial	Partial
141	0.47	No		Partial	Partial
142	0.53	No		Partial	Partial
143	0.45	No		No	No
144	0.50	No		No	Partial
145	0.52	Yes		No	No
146	0.14	Yes		No	No
147	0.18	Yes		No	No
148	0.22	Yes		No	No
149	0.10	Yes		No	No
150	0.13	Yes		No	No
151	0.41	No		Full	Partial
152	0.64	No		No	Full
153	0.63	No		Partial	Full
154	0.96	No		Full	Full
155	0.99	No		Full	Full

Table 2. Data of daily mean VPD and rainfall for 2020.

DOY	Daily Mean of VPD (kPa)	Rain Fall	Hysteresis Curve
114	0.28	No	Partial
115	0.73	No	Partial
116	0.83	No	No
117	0.61	No	No
118	0.67	No	No
119	0.37	Yes	No
120	0.54	Yes	No
121	0.7	No	No
122	0.8	Yes	No
123	0.59	No	No
124	0.38	Yes	Partial
125	0.74	No	Partial
126	1.06	No	No
127	0.26	No	No
128	0.53	Yes	Partial
129	0.96	No	No
130	0.78	No	No
131	0.51	No	Partial
132	0.71	No	No
133	0.7	Yes	No

Table 2. *Cont.*

DOY	Daily Mean of VPD (kPa)	Rain Fall	Hysteresis Curve
134	0.49	Yes	Partial
135	0.39	No	Partial
136	0.88	No	Partial
137	0.41	Yes	No
138	0.34	No	Partial
139	0.31	No	No
140	0.38	Yes	Partial
141	0.15	Yes	No
142	0.6	Yes	Full
143	0.89	No	Full
144	1.47	No	Full
145	0.47	Yes	No
146	1.02	No	Partial

Table 3. Frequency of observations showing hysteresis curves in fruits 2, 3 and 4 in the first period of study. Pearson test was not significant (p-value > 0.05).

Hysteresis Curve	Frequency
No hysteresis	0.573
Partial	0.427
Full	-
Pearson test value	0.204

Table 4. Frequency of the observations showing hysteresis curves in fruits 2, 3 and 4 in the second period of study. Asterisk indicates that Pearson test was significant (p-value < 0.05).

Hysteresis Curve	Frequency
No hysteresis	0.133
Partial	0.200
Full	0.667
Pearson test value	0.0224 *

On the other hand, fruit 1 showed a complete clockwise hysteresis curve (Figure 5) on 5 days out of 6 studied (from DOY 124 to DOY 129). On the last day (DOY 129), no hysteresis could be observed because during the second part of the day the transversal diameter did not increase (Table 3).

In the second period, the VPD increased to 0.74 ± 0.62 kPa (from 0.30 ± 0.62 kPa in the first period) in 2019 and to 0.89 ± 0.63 (from 0.58 ± 0.43 kPa in the first period) in 2020.

With the softening of the mesocarp, the pressure of the arms of the extensometer acted as a suppressing force (Figure 6). Thus, the transversal diameters of the fruits appeared to be decreasing. For this reason, although the curves did not close, we can consider them to exhibit hysteresis.

Thus, in two out of 15 total studied cases (5 days of the second stage for the three fruits) there was no hysteresis, in three out of 15 there was partial hysteresis, and in 10 out 15 there was full hysteresis (Tables 3 and 4). Full hysteresis delimited a greater area than partial hysteresis (Figures 5 and 7).

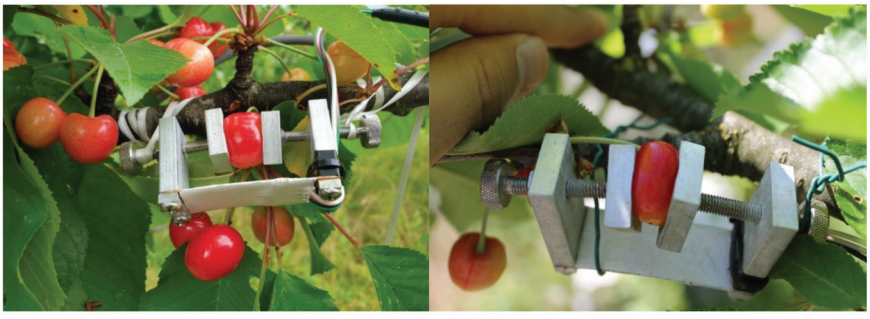

Figure 6. Cherry fruits on 28 May 2019 (DOY 148, **left**) and 22 May 2020 (DOY 143, **right**).

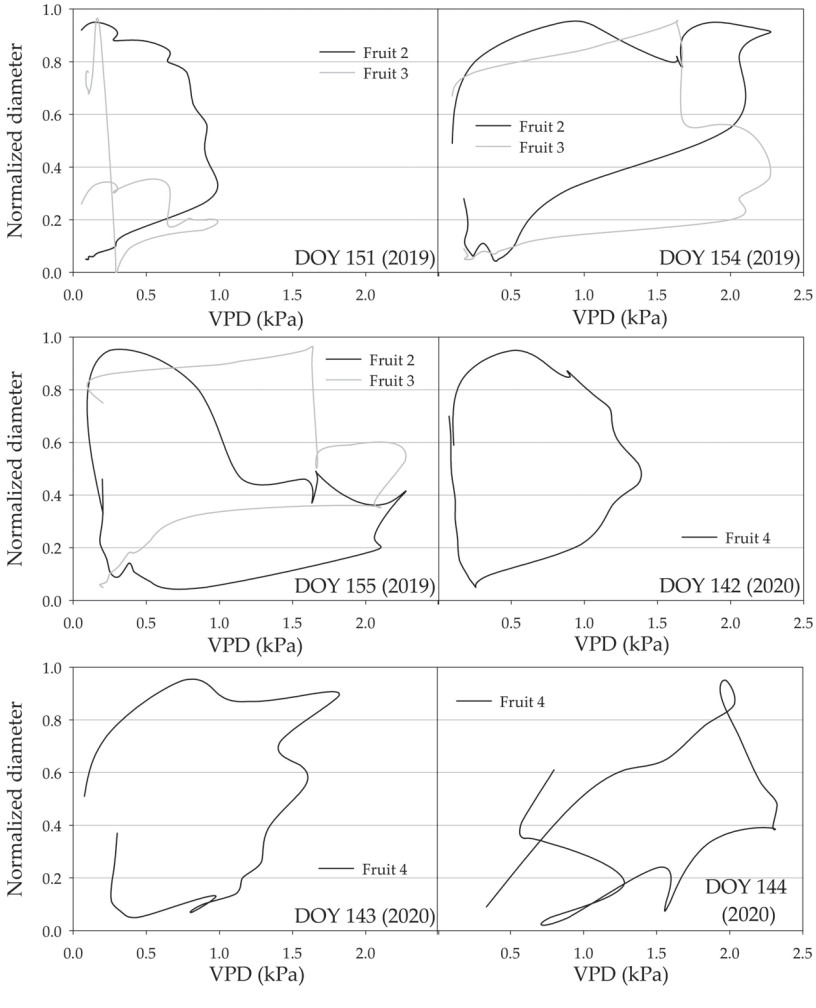

Figure 7. Most representative relations between VPD and transversal diameter in fruit growth stage 4 in 2019 and 2020.

4. Discussion

The results of this study showed that VPD can influence daily fruit growth and even induce full clockwise hysteresis during maturation. It is likely that the maturation date for the cherry fruit can be anticipated if the average VPD is higher, as it was in 2020, when the growth season was shorter than in 2019.

Generally, growing fruits can recover the shrinkage which was recorded in the early morning due to the VPD increase during the afternoon and the night, when the VPD is lower. On the contrary, fruits that are close to drop-down (for example, fruit 1) undergo a size reduction without early recovery, possibly due to the loss of inflow capacity from the tree [12] and related to abscising set formation between the fruit and the pedicel.

Therefore, the difference between growing fruit (fruits 2, 3 and 4) and dropping fruit (fruit 1) was observable when the VPD increased from the sunrise. With daily increases in VPD, fruits 2, 3 and 4 showed a short period of diameter decrease and then a stability phase in terms of diameter. However, in fruit 1, the diameter stability phase was not detectable. On the other hand, all fruits showed strong growth during the VPD decrease from sunset to sunrise.

Rain can change the diameter growth trend during the daylight phase, causing a decrease in VPD and a boost in the fruit diameter, likely related to a water boost, as was found by Correia et al. [8]. This phenomenon depends on the intensity of rain, the rainy hours and the circadian time of the rain.

During the third fruit growth period, the relation between VPD and transversal diameter showed partial hysteresis in the daily phases between sunrise and sunset. From sunset, the transversal diameter grew exponentially as the VPD decreased, defined by a power trend of the curve.

The behavior of fruit 1 was also observable in fruits 2, 3 and 4 during the second period of the study (the maturation stage). In this stage, the mean hourly VPD increased in both years. Brüggenwirth et al. [12] demonstrated that transpiration flow in sweet cherry fruit is closely linked to VPD throughout development. The dependence on the VPD increases because the stomata lose their functionality during stage three [30]. During this stage, it can be hypothesized that the xylem inflow equalizes the outgoing transpiration outflow, but in late stage three (around the beginning of second study period) the water uptake from the xylem to the fruit is getting close to zero [12,31,32]. The VPD increase during the day, together with the non-functionality of some stomata, plus the stopping of the xylem conductivity, may have caused the final size decrease observed in the fruits in 2019, and hampered growth in 2020.

In addition, during stage four of fruit development, the softening of the mesocarp increased [29] and the fruit was unable to oppose the pressure of the extensometer arms. In this period, the limited phloem connection may still help recover the water during the night, and thus the daily fruit growth trend realized a full hysteresis curve, in which the return curve did not overlap with the forward curve, nor did it pass close to it. In the case of partial hysteresis, the outward and return curves were very close.

Considering the results for 2019 and 2020, it can be hypothesized that a fully clockwise hysteresis curve is observable only when cherry fruits are fully mature (Tables 3 and 4). Thus, the mature fruit can exhibit large differences in size during the day, depending on VPD [12].

5. Conclusions

In this study, we investigated the continuous transversal diameter growth of sweet cherry fruits in 2019 and 2020, and considered its relationship with VPD. The results showed that on some random days of the third stage of fruit growth, the response of fruit transversal diameter versus VPD demonstrated a partial clockwise hysteresis curve. Only in the fourth stage of fruit growth was fully clockwise hysteresis detectable. Thus, the detection of partial and fully clockwise hysteresis curves can be considered a useful tool

in monitoring the real phenological stages of fruit and can be used as a basis to develop precision farming techniques applied to decision support systems (DSSs).

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Characterization of Japanese Apricot (*Prunus mume*) Floral Bud Development Using a Modified BBCH Scale and Analysis of the Relationship between BBCH Stages and Floral Primordium Development and the Dormancy Phase Transition

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Abstract: Bud dormancy is an important developmental stage that ensures that trees can tolerate environmental stresses in winter and bloom uniformly in the following spring. Regarding Rosaceae floral buds, exposure to chilling conditions promotes floral primordium development and the transition from endodormancy to ecodormancy. A subsequent period of warm conditions induces blooming. In Japanese apricot (*Prunus mume*), dormancy progression is accompanied by morphological changes that alter the bud appearance and internal structures. We used a modified BBCH scale and conducted microscopy analyses to elucidate the bud developmental stage of three cultivars with contrasting chilling requirements. The floral bud developmental period corresponding to BBCH stages 51–53 includes the transition from endodormancy to ecodormancy in all three cultivars. Male meiosis and microspore development occurred during this transition in high-chill cultivars, but were detected considerably later than the transition in the low-chill cultivar. A slow or suspended developmental phase was observed only for the high-chill cultivars upon completion of floral primordium organ differentiation, suggesting that chilling may be required to induce floral bud maturation and dormancy release only in high-chill cultivars. Possible relationships among BBCH stages, flowering-related morphological characteristics, and the dormancy phase transition in Japanese apricot are discussed.

Keywords: chilling requirement (CR); floral bud; dormancy; microsporogenesis; relative growth rate (RGR); BBCH scale

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1. Introduction

Exposure to low temperatures affects floral development in numerous species. A prolonged period of low temperatures is necessary in bulb species for normal floral organ development; otherwise, floral organs will be severely deformed [1]. Therefore, it is considered that the chilling treatment that promotes floral primordium development and maturation is necessary for the subsequent blooming and fruiting process [2]. Strictly speaking, bud dormancy refers to the state in which the bud meristem is unable to resume growth and development under suitable environmental conditions [3]. Lang (1987) divided fruit tree bud dormancy into endodormancy and ecodormancy. During endodormancy, bud growth ceases because of internal factors, and a particular period of low temperatures is required for buds to regain the competency to grow. In contrast, in ecodormancy, buds can resume growth, but unfavorable environmental conditions will arrest or delay active growth [4]. Buds shift from endodormancy to ecodormancy when the cultivar-dependent chilling requirement (CR) is fulfilled. Moreover, a subsequent exposure to a certain period of warm conditions is required to release buds from ecodormancy so they may enter the full bloom stage. If the floral buds of Rosaceae species, including Japanese apricot (*Prunus mume*), are not sufficiently exposed to low temperatures, they will be unable to develop

further, resulting in a relatively low blooming percentage [5]. Thus, low temperatures contribute to floral development and dormancy release.

Regarding *Prunus* fruit trees, although their floral buds do not exhibit high blooming competency during endodormancy, they apparently continue to develop during endodormancy and ecodormancy [2,6]. Sweet cherry (*P. avium*) and apricot (*P. armeniaca*) floral buds remain in a quiescence stage (i.e., rest) during dormancy until the CR is fulfilled, after which they resume developing [2,6]. Endodormancy release and ecodormancy release are accompanied by morphological changes affecting the appearance and internal structures of buds (e.g., bud scale color and pollen development) [2,7–10]. The Biologische Bundesanstalt, Bundessortenamt and CHemical industry (BBCH) scale systematically defines the botanical characteristics of plant developmental stages [11]. In the genus *Prunus* of the family Rosaceae, researchers have used BBCH scales to characterize sweet cherry (*P. avium* L.), apricot (*P. armeniaca* L.), and almond (*P. dulcis* L.) at different stages of growth and development [7,12,13]. Additionally, BBCH scales have been applied to assess the relationship between the sweet cherry bud appearance and dormancy status [2,14]. In sweet cherry, BBCH stage 5 (reproductive development) was further defined using dormancy characteristics. For example, BBCH stage 50 represents deep dormancy, whereas BBCH stage 51 involves the initiation of dormancy release [2,7,14]. However, the relationship between the BBCH scale and the dormancy status remains to be elucidated in other *Prunus* species.

Among the histological changes during floral bud development, microsporogenesis is an important event associated with the dormancy status of *Prunus* species [2,6,14,15]. The timing of microsporogenesis is reportedly correlated with different CRs and blooming dates among apricot (*P. armeniaca*) and sweet cherry (*P. avium*) cultivars [2,14,15]. In apricot, after the CR of the floral buds has been fulfilled, the pollen mother cells are reactivated, which coincides with BBCH stage 53 [6,15]. In sweet cherry, however, male meiosis is induced after exposure to warm conditions following CR fulfillment [16].

The objective of this study was to evaluate the potential relationship between morphological characteristics and the dormancy phase transition in Japanese apricot floral buds. Accordingly, we determined the timing of the transition from endodormancy to ecodormancy in three cultivars with contrasting blooming dates. To clarify floral bud growth characteristics, relative growth rate (RGR) and water content were investigated. Previous studies revealed a relationship between water content trends and dormancy progression in several Rosaceae fruit trees [2,17]. We also described Japanese apricot floral bud's phenological stages using a modified BBCH scale for the first time and observed the internal development of floral organs. Here, we discuss the relationship between morphological development and dormancy phase transition in *Prunus* fruit trees.

2. Materials and Methods

2.1. Plant Materials

Adult trees (>15 years old) of 19 Japanese apricot cultivars grown at the Kyoto farmstead of the experimental farm of the Graduate School of Agriculture, Kyoto University (35.032 N, 135.785 E), Kyoto, Japan, were used in this study. We determined the blooming date (BD) of each cultivar in the 2019–2020 season. BD was defined as the date when 50% of the floral buds on a tree were in the original BBCH stage 57 [11], with visible petal tips in the field. The temperature was recorded hourly using the T&D Thermo Recorder, TR-50U2 (T&D Corporation, Matsumoto, Japan).

2.2. Evaluation of Growth/Dormancy Status

To investigate the timing of the dormancy phase transition in the 2019–2020 season, bud break competency was tested as described by Fadón et al. [18]. Branches were collected on the 25th of each month from three cultivars with contrasting blooming dates ('Nanko', 'Shirokaga', and 'Ellching') and separated into several pieces (single-node cuttings), and a total of five replicates and 15 pieces from each replicate were used. Then, single-node cuttings were incubated under forcing conditions (18 h day/6 h night, 23 °C). The bud

break rate was recorded twice weekly until 3 weeks after initiating the incubation. In the 2020–2021 season, bud break competency was tested in 'Nanko' using branches collected on 30 November and 31 December. We also calculated the RGR and water content to compare seasonal bud growth patterns among the three cultivars. A total of four replicates were used in the RGR calculation, and each replicate included 20 buds. The RGR was calculated using the following formula:

$$\mathrm{RGR} = \frac{\mathrm{W2} - \mathrm{W1}}{(\mathrm{T2} - \mathrm{T1}) \cdot \mathrm{W1}}$$

where W1 is bud fresh weight at time point 1 (g), W2 is bud fresh weight at time point 2 (g), T1 is time point 1 (day), and T2 is time point 2 (day).

The collected buds were lyophilized and weighed. A total of four replicates were used in water content (%) calculation, and each replicate included 20 buds. The water content was calculated using the following formula:

$$\text{Water content (\%)} = (\text{fresh weight} - \text{dry weight})/\text{fresh weight} \times 100.$$

2.3. Estimation of the CR for Endodormancy Release and the Heat Requirement (HR) for Blooming

The date of transition from endodormancy to ecodormancy (i.e., the date of CR fulfillment) was defined as the date when the collected branches had a 50% bud break rate under forcing conditions, which was estimated by a linear regression of increasing seasonal bud break competency determined using the above-described method. We used the following three models for evaluating the CR: (1) chilling hour model [19], (2) Utah model [20], and (3) dynamic model [21]. Regarding the chilling hour model, all hours with temperatures between 0 and 7.2 °C were considered chilling hours [19]. For the Utah model, the chill unit (CU) for hourly temperatures was calculated using the conversion table of Utah CUs [20]. The dynamic model was applied using a program available on the University of California Division of Agriculture and Resources website (https://ucanr.edu/sites/fruittree/How-to_Guides/Dynamic_Model_-_Chill_Accumulation/).

The growing degree hour (GDH) value was assigned for all hours with temperatures between 4.5 and 25 °C, and was calculated as the temperature minus 4.5 °C. The HR was calculated as the accumulated GDH values from the CR fulfillment date to the BD [22,23].

2.4. Analyses of Bud Phenology and Internal Floral Primordium Development

The floral buds of the three cultivars grown in the field were photographed twice monthly (on average) from September to February in the 2019–2020 season. The 'Nanko' floral buds were also photographed weekly in December during the 2020–2021 season. Floral bud samples were collected every 2 weeks in the 2019–2020 season (28 September, 14 October, 29 October, 15 November, 30 November, 15 December, and 30 December) and were preserved in FAA fixative solution (formaldehyde/alcohol/acetic acid/H_2O = 2:10:1:7). After replacing the fixative solution with a series of sucrose solutions (10%, 20%, and 30%) for 2 h each, the samples were embedded in Super Cryoembedding Medium (Leica Microsystems GmbH, Wetzlar, Germany) and then frozen using isopentane as the refrigerant in liquid nitrogen. For each sample, 7–15 μm longitudinal sections were prepared using a cryostat microtome (CM1520, Leica) and stained with toluidine blue O solution (10 mM benzoic acid, 10 mM sodium benzoate, and 2 mM toluidine blue O). The floral bud sections were examined using the DP80 light microscope (Olympus, Tokyo, Japan) and photographed.

3. Results

3.1. Contrasting the CR and HR of Three Cultivars

On the basis of the distribution of the BD, 19 cultivars from the experimental farm of Kyoto University were classified as early blooming, medium blooming, late blooming, and extra late blooming (Figure 1; Table 1). In this study, one representative cultivar was

selected from each blooming date category, with the exception of medium blooming, and used for the analyses of morphology, organ development, and dormancy status. The BD of 'Ellching' was 6 January 2020, whereas it was 4 and 10 February 2020 for 'Nanko' and 'Shirokaga', respectively.

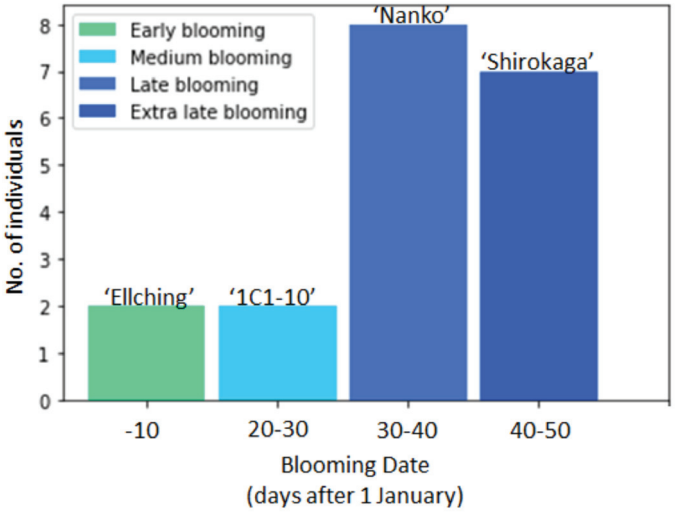

Figure 1. Frequency distribution of blooming dates in the 19 Japanese apricot collections during the 2019–2020 season. 'Ellching', early blooming; '1C1-10', medium blooming; 'Nanko', late blooming; 'Shirokaga', extra late blooming.

Table 1. Blooming date (BD) for 19 Japanese apricot cultivars in the 2019–2020 season.

Cultivar	BD2020 [1]	Origin
Ellching	6	Taiwan
Hayazakinanko	7	Japan
1C1-10	24	Japan
Kairyo-uchida	28	Japan
Ryukyokoume	32	Japan
1KO-26	33	Japan
Benisashi	34	Japan
Nanko	35	Japan
Kotsubnu-nanko	35	Japan
Koshinoume	35	Japan
Kagajizo	37	Japan
Kensaki	38	Japan
Shirokaga	41	Japan
Hachiro	47	Japan
Rinshu	49	Japan
Koshukoume	49	Japan
Aojiku	49	Japan
Oshuku	49	Japan
Gyokuei	49	Japan

[1] BD2020: blooming date was calculated from 1 January 2020 (Julian days).

On the basis of the seasonal changes in bud break competency, the CR fulfillment dates were estimated to be 12 November, 29 December, and 8 January for the early-blooming 'Ellching', late-blooming 'Nanko', and extra-late-blooming 'Shirokaga', respectively (Figure 2). Using the chilling hour model (indicated by CH) [19], the Utah model

(indicated by CU) [20], and the dynamic model (indicated by CP) [21], the CRs were calculated as follows: 'Ellching', 2 CH, 5.5 CU, and 2 CP; 'Nanko', 395 CH, 584.5 CU, and 31.5 CP; and 'Shirokaga', 519 CH, 754.5 CU, and 39.3 CP. The starting point of chilling temperature accumulation was estimated from 9 November, 8 November, and 6 November for the chilling hour model, the Utah model, and the dynamic model, respectively. The results of the three models reflect the differences in the early and late blooming dates (i.e., the extra-late-blooming cultivar had the highest CR) (Figure 3). Therefore, we defined 'Ellching', 'Nanko', and 'Shirokaga' as low-, high-, and extra-high-chill cultivars, respectively. The analysis using the GDH model revealed that 'Ellching' and 'Shirokaga' had the highest (6294.4) and lowest (2436.8) GDH values, respectively (Figure 4).

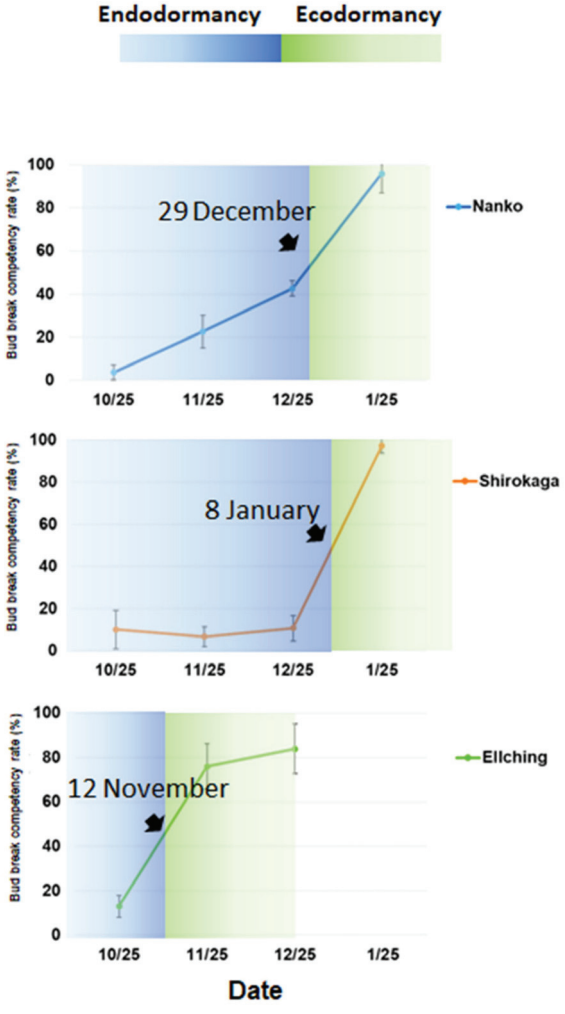

Figure 2. Seasonal changes in the bud break competency of the floral buds of three cultivars. The CR fulfillment dates were estimated to be 29 December, 8 January, and 12 November for the late-blooming 'Nanko', the extra-late-blooming 'Shirokaga', and the early-blooming 'Ellching', respectively. Five replicates were analyzed, and 15 nodes were examined for each replicate. The error bars represent the SE.

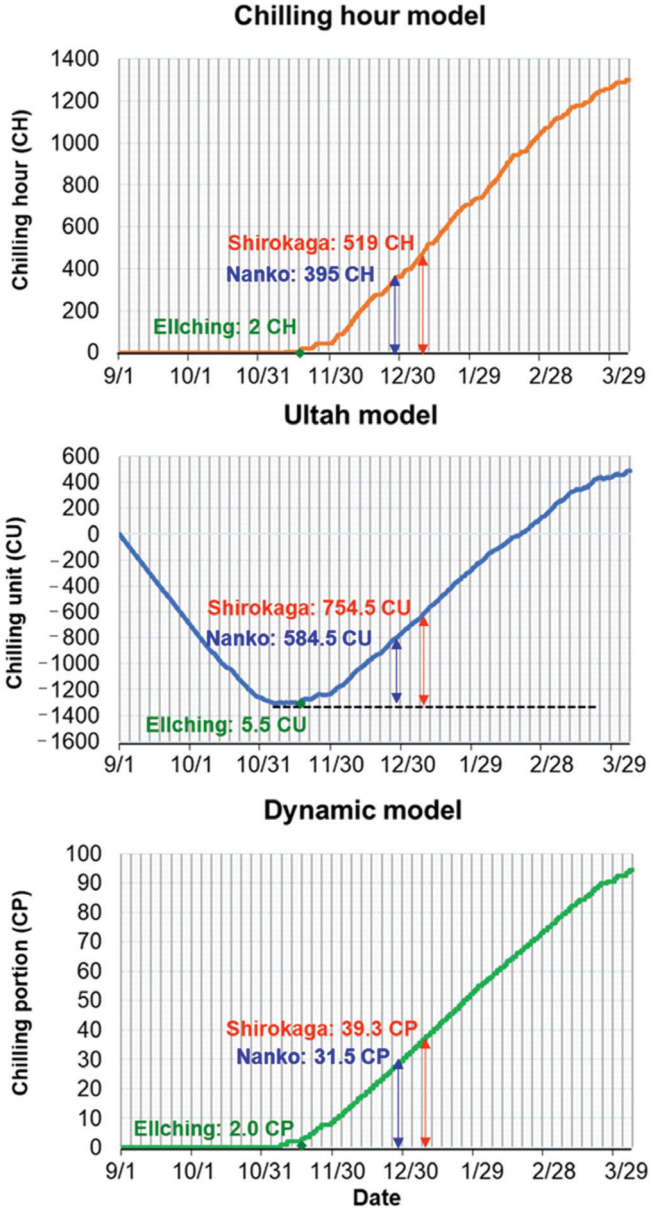

Figure 3. Estimation of the CR of three cultivars using the chilling hour model (top), the chill unit (Utah) model (middle), and the dynamic model (bottom). 'Nanko', high chill; 'Shirokaga', extra high chill; 'Ellching', low chill.

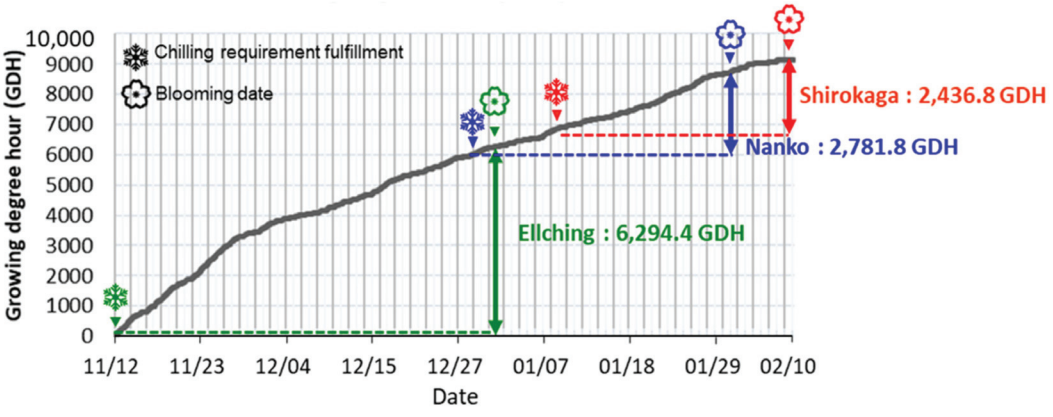

Figure 4. Evaluation of the HR of three cultivars using the growing degree hour (GDH) model. 'Nanko', high chill; 'Shirokaga', extra high chill; 'Ellching', low chill. Ice crystal mark means chilling requirement fulfillment; flower mark means blooming date.

3.2. Seasonal Changes in the RGR and Water Content of the Low-Chill and High-Chill Cultivars

An examination of the floral bud development of all tested cultivars revealed a low RGR from September to October (Figure 5a). The low-chill cultivar ('Ellching') grew rapidly (0.14 $g \cdot g^{-1} \cdot day^{-1}$) from November to December. In contrast, the high-chill and extra-high-chill cultivars had a relatively stable RGR (0.05–0.08 $g \cdot g^{-1} \cdot day^{-1}$) from November to January. The floral bud water content increased consistently during dormancy progression (from 30% to 80%) (Figure 5b), with a similar trend detected for all three cultivars.

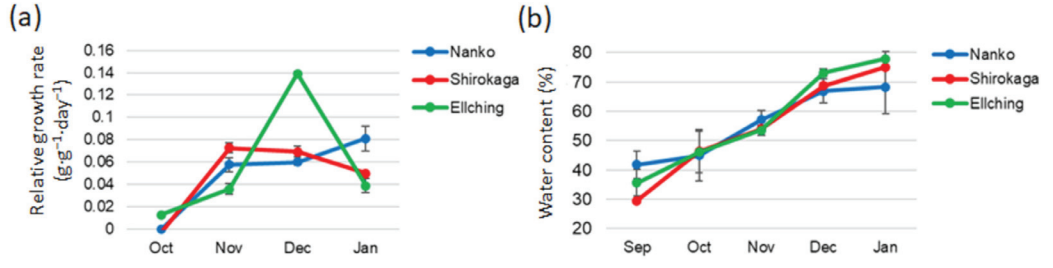

Figure 5. Seasonal changes in the relative growth rate and the water content during dormancy in 2019–2020. (**a**) Relative growth rate. (**b**) Water content (%). 'Nanko', high chill; 'Shirokaga', extra high chill; 'Ellching', low chill. Four replicates were analyzed, and 20 flower buds were examined for each replicate. The error bars represent the SE.

3.3. Establishment of a Modified BBCH Scale for Japanese Apricot Floral Buds

To establish a BBCH scale for Japanese apricot floral buds, we modified the original BBCH scale [11]. More specifically, because Japanese apricot does not form inflorescences, we omitted the description of stage 55 (single flower buds visible (still closed) borne on short stalks) from the BBCH scale. In the modified BBCH scale, 'principal growth stage 5: flower emergence' was used to further describe the floral bud development from dormancy to blooming in the three tested cultivars (Figure 6). Both 'Nanko' and 'Shirokaga' remained in BBCH stage 50 from September to November (Figure 6c), whereas 'Ellching' transitioned into stages 51–53 in late October (Figure 6c). In December, the 'Nanko' and 'Shirokaga' floral buds were in BBCH stages 51–53. In contrast, most 'Ellching' floral buds were in BBCH stages 54–56. Additionally, the 'Nanko' floral bud appearance underwent similar changes in BBCH stages 51–53 in December during the 2019–2020 and 2020–2021 seasons

(Figure 7). Most 'Nanko' and 'Shirokaga' floral buds were in BBCH stages 54–56 until early January when they transitioned into BBCH stages 57–59 (i.e., blooming period). The 'Ellching' floral buds remained in BBCH stages 54–56 for a longer period from December to early January, after which they entered the blooming period.

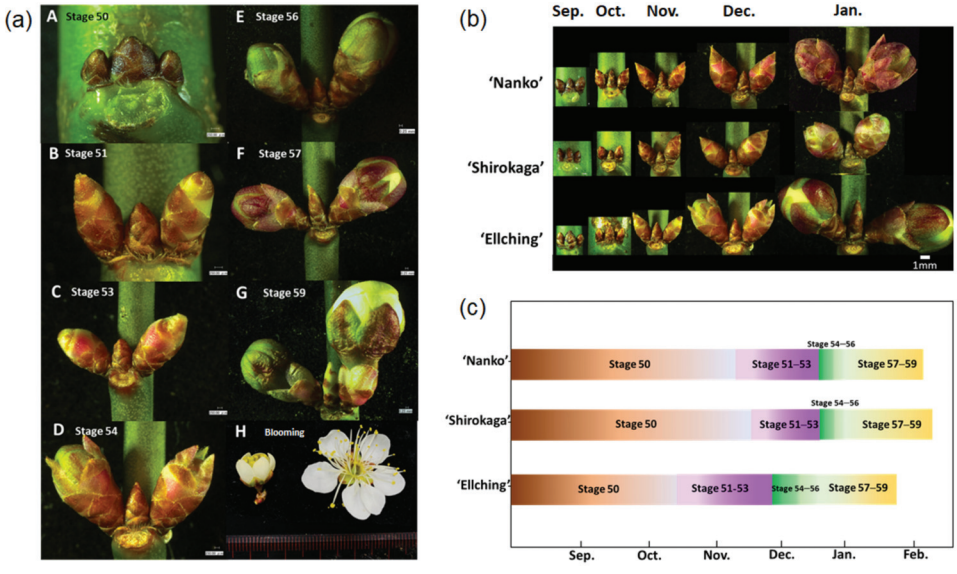

Figure 6. Evaluation of three Japanese apricot cultivars during dormancy using the modified BBCH scale. (**a**) Floral bud developmental stage. Scale bar = 250 μm. (**b**) Seasonal changes in the bud appearance among Japanese apricot cultivars with varying blooming dates. Scale bar = 1 mm. (**c**) BBCH scheme of three Japanese apricot cultivars during dormancy. 'Nanko', high chill; 'Shirokaga', extra high chill; 'Ellching', low chill.

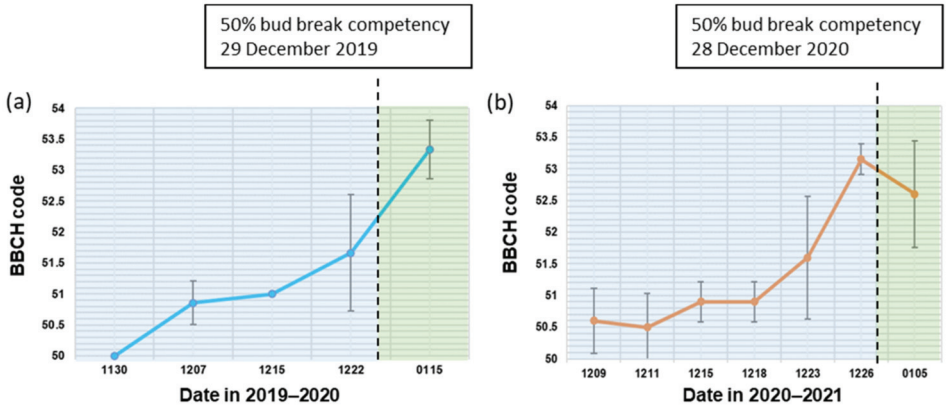

Figure 7. BBCH scale evaluation of 'Nanko' floral buds during December in 2019–2020 and 2020–2021 seasons: (**a**) 2019–2020, (**b**) 2020–2021. Ten flower buds from each stage were analyzed in 2019/2020 and 20 flower buds were examined from each stage. The error bars represent the SE. Dash line means the time point of 50% bud break competency.

3.4. Comparison of Seasonal Floral Primordium Development among Cultivars with Contrasting CRs

The floral initiation (initiation of floral organ formation) of the low-chill cultivar ('Ellching') was induced relatively late, but the floral organs rapidly formed by late October (Figure 8a). The floral primordium developed earlier in the high-chill cultivars than in 'Ellching', and pistil formation was completed by the end of October. The timing of pollen maturation differed between 'Ellching' and the high- and extra-high-chill cultivars (Figure 8b). Degraded tapeta and mature pollen were detected in anthers on 15 December for 'Ellching', but on 30 December for 'Nanko' and 'Shirokaga'.

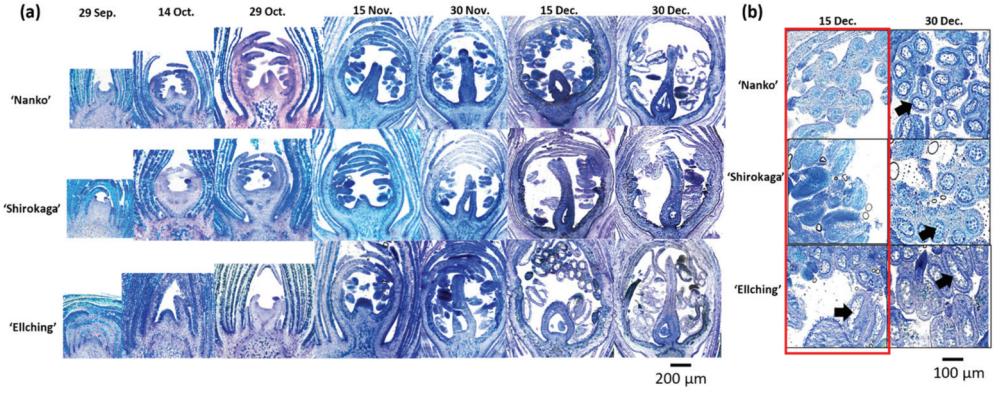

Figure 8. Longitudinal sections of the floral buds from different Japanese apricot cultivars during dormancy. (**a**) Floral organ: A, anther; C, carpel; P, petals; O, ovary. Scale bar = 200 μm. (**b**) Differences in pollen developmental status among cultivars. Scale bar = 100 μm. 'Nanko', high chill; 'Shirokaga', extra high chill; 'Ellching', low chill.

3.5. Correlation between BBCH Stages and the Floral Bud Physiological Dormancy Status

The results of a Pearson correlation analysis indicated that BBCH stages were highly positively correlated with bud break competency, RGR, and water content (correlation coefficients: 0.88, 0.51, and 0.82, respectively) (Figure 9).

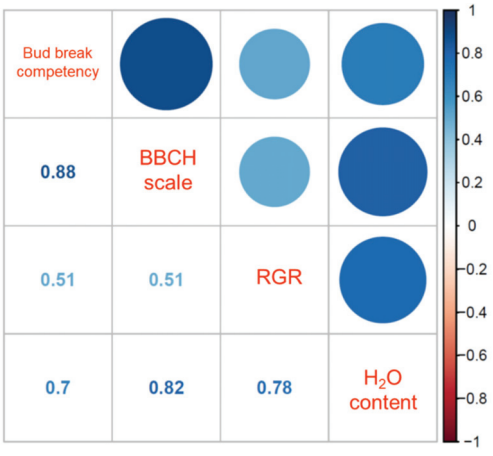

Figure 9. Pearson correlation matrix of traits during dormancy. The color scale reflects the correlation, with 1 indicating a completely positive correlation (dark blue) and −1 indicating a completely negative correlation (dark red) between two traits.

4. Discussion

4.1. Comparison of the Japanese Apricot Floral Bud Dormancy Progression of Cultivars with Different Blooming Dates

Seasonal changes in bud break competency suggest that earlier and later endodormancy release may be primarily responsible for the earlier and later blooming of 'Ellching' and 'Shirokaga', respectively, compared with 'Nanko'. Thus, we categorized 'Ellching', 'Nanko', and 'Shirokaga' as low-chill, high-chill, and extra-high-chill cultivars, respectively. We evaluated the growth/dormancy physiological status of these cultivars by analyzing the water content and RGR. Earlier research proved that the floral bud water contents of sweet cherry [24] and Japanese pear (*Pyrus pyrifolia*) [25,26] increase gradually from the dormancy stage to the blooming stage. Moreover, the free water content is reportedly correlated with floral primordium development and endodormancy release in Japanese pear [25]. In the current study, the water contents of all cultivars increased until the blooming stage. However, no clear difference was observed between the low-chill and high-chill cultivars. Additionally, for all cultivars, the RGR was lowest in October. The high-chill cultivars had a constant RGR from November onward, whereas the RGR of the low-chill cultivar increased substantially in December. These results suggest that floral bud growth ceased in October, but resumed somewhat from November onward as dormancy progressed in all three cultivars. This is consistent with the results of a previous study, in which the floral development of Rosaceae fruit trees continued during dormancy [5]. Floral organ formation was observed in October and was completed by the end of October in all three cultivars. Additionally, endodormancy was released in early November, late December, and early January in the low-chill, high-chill, and extra-high-chill cultivars, respectively. Therefore, for 'Ellching', there was either no time or a very short period after floral organ formation before endodormancy release. In contrast, 'Nanko' and 'Shirokaga' had a slow or suspended developmental phase that lasted approximately 2 months before endodormancy release. We hypothesize that the CR of the low-chill cultivar reflects the low-temperature requirement for organ development and floral bud maturation, whereas the CRs of the high-chill cultivars reflect the low-temperature requirement for floral bud maturation and the release of the slow or suspended developmental phase.

4.2. Calculated CRs and HRs for Japanese Apricot Floral Buds

In this study, the CRs calculated using three different models confirmed that 'Ellching' is a low-chill cultivar and 'Nanko' is a high-chill cultivar, which is consistent with our previous findings [27,28]. However, the CRs indicated by CH determined in this study are not identical to those calculated by Yamane et al. (2006). More specifically, the CRs for 'Nanko' and 'Ellching' floral buds were approximately 500 and 300 CH, respectively, in the earlier study [27], whereas they were lower in the current study (i.e., 395 and 2 CH, respectively). The methodology for CR calculation was not exactly the same between these two studies, which may affect inconsistency in CR values. These results also imply that the genotype-dependent CR might not be precisely determined by the chill hour model. Alternatively, even though the CR is genotype dependent and genetically controlled, it varies depending on the environmental condition and tree age. Additionally, an extremely low CR value of 'Ellching' estimated in this study may raise the question of whether 'Ellching' is "low chill" or "no chill" for endodormancy release.

Regarding the HR, the GDH model indicated that 'Ellching' (low-chill cultivar) had a higher GDH value (6294) than 'Nanko' (2781) or 'Shirokaga' (2436) (Figure 4). The higher GDH value for 'Ellching' may reflect the longer ecodormancy period for this cultivar, with 56 days between the dormancy transition date and the blooming date, unlike the 36 and 33 days between these two dates for 'Nanko' and 'Shirokaga', respectively. Similar results were obtained for the HR estimated using the GDH model in apricot. Although the GDH value of the apricot cultivar 'Penta' (8923) was higher than that of 'Tardona' (7789), 'Tardona' bloomed approximately 10 days later than 'Penta' ('Penta', 12 March; 'Tardona', 21 March) [29]. Considering that a prolonged chilling period reportedly de-

creases the HR for blooming in peach [30], a mutual compensation between chilling and warm temperature requirements for blooming may occur. This leads to the question of whether the higher GDH value for the low-chill cultivar may reflect a greater HR, namely, lower sensitivity to warm conditions, or the HR may not be accurately determined using the GDH model. Alternatively, the HR initiation date determined on the basis of bud break competency (>50% bud break under forcing conditions) may be inappropriate for estimating the genotype-dependent HR, especially for low-chill cultivars. Current models for estimating the dormancy status and blooming mostly focus on temperature alone. Moreover, dormancy induction is regulated by multiple environmental factors in *Prunus* species (e.g., the photoperiod, temperature, and water deficit stress) [31–33]. Thus, dormancy progression and release as well as blooming may be regulated by temperature and other environmental factors. Using their developmental rate model for predicting the CR and HR, Kitamura et al. (2017) concluded that warm conditions during ecodormancy may be the primary factor explaining the year-dependent blooming date of 'Nanko', rather than cold conditions during endodormancy [28]. Collectively, flowering time (blooming) models, especially for HR predictions, must be improved for future dormancy studies and for practical use.

4.3. Relationship between the Dormancy Phase Transition and BBCH Stages in Japanese Apricot

In this study, we established a BBCH scale applicable for Japanese apricot and compared it with the physiologically determined dormancy status. We revealed that BBCH stage 50 represents deep dormancy. In this stage, floral buds exhibit relatively low bud break competency, and the dormant buds are covered by dark brown scales (Figure 6). Previous investigations indicated that BBCH stage 50 corresponds to deep dormancy in sweet cherry [2,7]. During BBCH stage 50, floral organ primordia differentiated, and the stigma, anthers, and pistil were detectable (Figures 6 and 8). In BBCH stage 51, endodormant floral buds before CR fulfillment were swollen and covered by light brown scales (Figure 6; Table 2). In 'Nanko', bud break competency started to increase in BBCH stage 51. Additionally, BBCH stages 51–53 corresponded to the dormancy phase transition period (i.e., from endodormancy to ecodormancy), whereas BBCH stages 56–59 represented the blooming period, during which the bud break rate approached 100% under forcing conditions (16 h day/8 h night, 23 °C) (Figures 2 and 6). BBCH stages 51–53 corresponded to dormancy phase transition in the next growing season in 'Nanko', which supported the idea that the stages may represent dormancy transition (Figure 7). Our data indicated that the bud break competency was highly correlated with the BBCH stages (correlation coefficient of 0.88) (Figure 9), suggestive of a potential relationship between the physiological dormancy status and the apparent floral bud morphological changes in Japanese apricot.

Table 2. Description of the modified BBCH scale for Japanese apricot floral buds from dormancy to blooming.

	Principal Growth Stage 5: Flower Emergence
50	Floral bud closed, dark brown scales visible
51	Floral bud swelling, bud still closed, light brown scales visible
53	Scales further separated, light green bud sections, and light purple or pink scales visible
54	Internal floral organ enclosed by light green sepals, round green sepals visible
56	Flower pedicel elongating, green sepals still closed showing a round shape
57	Sepals open: petal tips visible, flower with white petals (still closed)
59	Flowers with petals forming a hollow ball

4.4. Connection between Pollen Maturation and the Dormancy Status in Japanese Apricot

Microsporogenesis is a biological indicator of CR fulfillment and reflects the reactivation of cells following dormancy in apricot and sweet cherry [6,8,15,34,35]. Microspore formation coincides with BBCH stage 53 in sweet cherry and apricot [2,36]. Regarding the Japanese apricot cultivars examined in this study, the timing of pollen maturation

matched the timing of CR fulfillment in 'Nanko' and 'Shirokaga' (Figures 2 and 4), which is consistent with the findings of earlier sweet cherry and apricot studies [6,8,15,35]. However, for 'Ellching' (low-chill cultivar), there was approximately 1 month between CR fulfillment and pollen maturation (Figures 2 and 8). In the high-chill cultivars, tapetum degradation during the pollen maturation process was observed in BBCH stages 53 and 54, and BBCH stages 54–56 were shorter (corresponding to a lower GDH value) than in the low-chill cultivar. In earlier investigations of apricot and sweet cherry, the plants used for analyzing pollen mother cell development were mostly high-chill cultivars, including sweet cherries 'Burlat' (981 CU) and 'Bing' (1082 CU) [2,8] and apricot 'Moniqui' (1050–1150 CU) [6]. The low-chill cultivar 'Ellching' originated in Taiwan, which is in a tropical and subtropical region [37]. Additionally, its 5.5 CU calculated using the Utah model was substantially lower than the CUs of the other Japanese apricot cultivars (Figure 3). This suggests that pollen maturation may be useful as a biomarker of endodormancy release and CR fulfillment only for high-chill cultivars in *Prunus*.

5. Conclusions

New classifications and terminologies to describe plant dormancy at the cellular level were recently proposed by Considine and Considine (2016) [38]. On the basis of the classification by Considine and Considine (2016) and the fact that bud growth continued after pistil formation until blooming in Japanese apricot, we propose that Japanese apricot floral buds may not enter a dormancy period, but become quiescent (i.e., slow or suspended development). Hence, the floral bud dormancy process of Rosaceae fruit trees may need to be redefined and reclassified in future studies. Additionally, our results suggest that the cultivar-dependent HR calculation model will need to be improved. In this study, we developed a modified BBCH scale useful for examining Japanese apricot floral bud development. Furthermore, we proved that the BBCH stages are correlated with seasonal changes in bud break competency. Accordingly, BBCH scales may be useful for characterizing the dormancy (quiescence) status of Japanese apricot floral buds.

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Conflicts of Interest: The authors declare no conflict of interest.

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Post-Harvest Quality and Sensory Evaluation of Mini Sweet Peppers

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Abstract: Sweet pepper (*Capsicum annuum* L.) is one of the most consumed vegetables in the world, being recognized as a food with high nutritional value. Recently, the market for sweet and colorful mini peppers has increased, especially among the most demanding consumers in the novelties in vegetables and functional foods. In this sense, we evaluated mini sweet peppers genotypes (Akamu, Kaiki, Kalani, Kaolin e Moke from Isla® seeds) regarding the physical-chemical, nutritional and sensory analysis aspects. A wide variability was observed among genotypes, highlighting the Kalani genotype for total carotenoids, and the genotypes Akamu, Kaiki and Kaolin for phenolic totals content and antioxidant activity. Moke and Kaolin showed higher vitamin C content and fruit firmness. Based on sensory analysis, Kalani, Kaiki, Kaolin and Akamu obtained greater global acceptance. The genotypes can be considered an important marketing strategy of mini sweet peppers trade, associating different shapes, colors and nutritional quality.

Keywords: *Capsicum annuum* L.; functional food; pepper pre-breeding; horticulture; sensory analysis

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1. Introduction

According to the World Health Organization (WHO), non-communicable diseases (NTDs) are the leading causes of death in the world, with ~80% of these deaths occurring in low- and middle-income countries. At least, one third occur among individuals under 60 years, while in high-income countries this proportion is 13% [1]. The main risk factors associated with NTDs include inadequate diets, low nutrition, and lack of physical activity. On the nutritional issue, low consumption of fruits and vegetables is considered an important risk factor that has coantributed to the increase in the overall burden of chronic diseases [2]. It is estimated that up to 2.7 million lives could be saved each year if fruit and vegetable consumption were increased [3].

In Brazil, 55.7% of adults (18 years) are overweight. These, 19.8% have obesity associated an inadequate eating habits, such as high consumption of ultra-processed foods and low consumption of minimally processed and fresh foods [4]. Several factors are related to this inadequate eating habit, including economic barriers, lack of nutritional knowledge and awareness, dietary preferences, and cultural factors [5,6]. In this context, several public policies and innovations in the agricultural sector have been carried out in order to encourage the consumption of vegetables and fruits in the world [2,7].

Mini and baby vegetables are considered one of the alternatives to increase the healthy foods consumption, mainly for children and young people, because they provide greater

ease for consumption and many of them are tastier, making it more attractive both visually and by taste [8]. The horticultural sector is constantly subjected to a process of change that requires the implementation of strategies for innovation of its products. An alternative to diversify this production chain is the cultivation of mini-vegetables which has presented advantageous economic opportunities [7,9]. The popularization of mini-vegetables began in the 1990s in Europe and expanded throughout the world [8].

Mini vegetables can be obtained through genetic improvement or are reduced by processing, while baby vegetables are obtained by pre-harvesting the traditional-sized product [9]. The best known crops of this segment in the Brazilian market are the mini tomatoes (*Solanum lycopersicum* L.), mini carrots (*Daucus carota* L.), mini lettuce (*Lactuva sativa* L.) and baby leaf, which includes lettuce, watercress (*Nasturtium officinale* R.), beetroot (*Beta vulgaris* L.), arugula (*Eruca sativa* L.), among other species [9].

Sweet pepper (*Capsicum annuum* L.) is among the ten most consumed vegetables in Brazil and in the world [10,11]. In addition to the attractions such as color and aroma, this vegetable is well known for its chemical and nutritional properties. Its fruits are great sources of vitamins A, B, C and E, as well to bioactive compounds with antioxidant activity like carotenoids and phenolic compounds [12,13]. Recently, mini sweet peppers of different colors, sizes and shapes have been introduced in the Brazilian market. However, there is lack of information about its physical-chemical and nutritional properties. Thus, the present work aimed to evaluate mini sweet peppers genotypes by physical-chemical, nutritional and sensory aspects.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiment was conducted at the Agronomy Department of the Universidade Estadual de Londrina (UEL), Londrina, Paraná, Brazil. Five genotypes of mini peppers (Akamu, Kaiki, Kalani, Kaolin and Moke) from the company Isla Sementes (Figure 1) were evaluated, which were sown in polystyrene trays, on Subras® commercial substrate. After 30 days, the seedlings were transplanted to pots, containing a mixture of soil and sand in a 3:1 ratio. The experimental design was complete randomized blocks with 3 replications and 12 plants per plot. The plants were kept in protected cultivation following the practices recommended for the cultivation of sweet peppers in Brazil. The fruits were harvested ripe (55 days after anthesis) and submitted to physical characterization. They were then stored under refrigeration at 4–6 °C for up to 3 days before biochemical analyses.

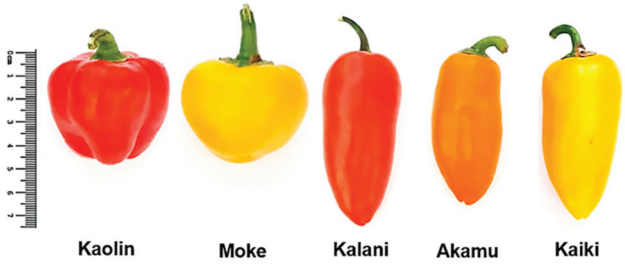

Figure 1. Photograph of the five mini sweet peppers evaluated in the present study.

2.2. Physical Characterization

Eight ripe fruit (55 days after anthesis) of each genotype were harvested and evaluated according to descriptors established by IPGRI [14], currently Bioversity International: length, diameter, pericarp thickness, mass and dry mass content. The color of the fruits was determined in a colorimeter using illuminant D65 (Minolta Co., Tokyo, Japan, model CR-13) by the luminosity, Chroma and Hue angle.

The firmness of five fruits of each genotype was determined in newtons (N) by the puncture test in a texturometer (Model TA. XT Plus, Stable Micro System, Surrey, UK). A needle probe was used to measure the resistance of the exocarp (skin) and pericarp. The puncture speed was 0.5 mm s^{-1} until 5 mm of the fruit was perforated in the equatorial zone of each fruit with no removed peel.

2.3. Biochemical Characterization

Soluble solids content (SS) was determined in a portable digital refractometer (Atago®) and expressed in °Brix. Titratable acidity (TA) was quantified by titrimetry based on AOAC method 942.15 [15] and expressed as% citric acid. Also was calculated the SS/TA ratio.

Vitamin C content was quantified by the titration method based on [15] and modified by [16], expressed as mg ascorbic acid 100 g^{-1}. Extraction of total carotenoids was adapted from Adalid, Roselló and Nuez [17] and quantification performed according to [18] using in a spectrophotometer (Genesys 10, Thermo, Waltham, MA, USA) at 450 nm, reporting as µg beta-carotene equivalents 100 g^{-1}.

For the quantification of phenolic compounds and antioxidant activity, an extraction was made from 1.0 g of fresh samples with 10 mL of 70% (*v*/*v*) ethanol, leaving the suspension under stirring for 30 min (Orbital-Nova Orgânica) at room temperature (25 °C). Then, the extract was centrifuged at 1013× *g* (Excelsa 2 Fanem model 205N) for 5 min and separated for analysis [18].

The extraction of total phenolic content, total flavonoid content and antioxidant activity was performed according to [18]. The quantification of phenolic compounds was based to [19], where Gallic acid was used as standard ranging from 10 to 100 mg L^{-1} (r = 0.9960) and expressed as mg gallic acid equivalents (GAE) per 100 g of fresh mass.

The antioxidant activity by sequestration of the 2,2-Diphenyl-1-picryl-hydrazyl (DPPH·) radical was performed according to Brand-Williams, Cuvelier and Berset [14]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard ranging from 0.20 to 1.00 mmol L^{-1} (r = 0.9992), and the result was expressed in % free radical scavenging. For the FRAP assay, 0.05 mL of the ethanolic extract were used, mixed with 1.0 mL of 80% (*v*/*v*) methanol and 1.0 mL of the FRAP reagent. The mixture was maintained at 37 °C for 30 min and the reading was performed at 595 nm, expressed as µmol TEAC per 100 g [20]. The ABTS test consisted of a mixture of 2 mL of the diluted ABTS•+ solution with 50 µL kept at darkroom temperature at 35 °C for 5 min. The absorbance was read at 753 nm. Trolox was used as standard and the result expressed as µmol of TEAC per 100 g [21].

2.4. Sensory Evaluation

The acceptance test was applied in a single session to a group of 155 evaluators (untrained volunteers), members of the university community of the Universidade Estadual de Londrina, Londrina, Paraná, Brazil. The proposal was approved by the Committee for Ethics in Research on Human Beings under registration CAAE 98049918.8.0000.5231.

The evaluators received one sample at a time, encoded with three random digits, served on a transparent disposable plate containing one fruit cut. The samples evaluation was done using a 10 cm hybrid hedonic scale anchored in the middle and extreme regions of the scale (0 = disliked extremely, 5 = neither liked nor disliked, 10 = liked extremely) (Villanueva et al., 2005) for the attributes: size, shape, color and overall acceptance. To verify the purchase intention of the product, a 5-point structured scale was used (1 = certainly would not buy, to 5 = certainly would buy).

2.5. Data Analysis

The data were submitted to the F-test ($p \leq 0.05$) by analysis of variance (ANOVA) and means were compared using the Tukey test ($p \leq 0.05$). Principal component analyzes (PCA) and Ward's hierarchical grouping were also performed. All statistical analyzes were performed using software R version 3.6.0 [22] using packages ExpDes [15] FactoMiner [16], pheatmap [17] and e ggplot2 [18].

3. Results

3.1. Physical and Biochemical Characterization

Analysis of variance showed a significant effect ($p \leq 0.05$) for all evaluated characteristics, indicating a high genetic variability. Genotypes varied in relation to fruit length (FL) from 44.07 to 82.40 mm. Regarding fruit diameter (FD), variation was observed between 25.23 and 38.03 mm, while pericarp thickness (PT) ranged from 2.60 to 4.37 mm.

Soluble solids (SS) content ranged from 5.87 to 9.97 °Brix, with the highest values observed for Kalani, Akamu and Kaolin. For titratable acidity (TA), Kalani genotype showed higher acidity, while Kaiki, Moke and Akamu had the lowest values. Regarding the evaluation of total carotenoid (CT) concentration, Kalani fruits also obtained the highest value (10323.08 mg beta carotene at 100 g^{-1}). For vitamin C (VITC), the values ranged from 183.82 to 242.65 mg of ascorbic acid in 100 g^{-1}, with the higher values observed in Moke, Kaolin and Kaiki with 242.65, 213.23 and 205.88 mg of ascorbic acid in 100 g^{-1}, respectively.

For phenolic total content (TPC), the values ranged from 677.2 to 825.5 mg GAE 100 g^{-1}, with the highest values observed for Akamu, Kaiki and Kaolin. These genotypes also showed high values for antioxidant activity (DPPH*, ABTS and FRAP methods). Regarding color, it is possible to affirm that Kaiki and Moke fruits are yellow, lighter and brighter, while Kalani and Kaolin fruits are red, darker and opaque than the others. The cultivar Akamu is characterized as bright orange fruits with intermediate luminosity to the yellow and red cultivars (Table 1).

Table 1. Physical, biochemical and sensory traits of five mini sweet pepper cultivars.

Traits [a]	Genotypes					CV% [b]
	Kalani	Kaiki	Akamu	Moke	Kaolin	
Physical						
FL	82.40 a	60.63 b	77.73 a	44.07 c	48.20 bc	9.5
FD	27.57 bc	25.87 bc	25.23 c	38.03 a	35.23 ab	11.9
PT	3.20 b	3.00 b	3.23 b	4.37 a	2.60 b	10.0
Biochemical						
SS	9.97 a	7.27 bc	8.70 ab	5.87 c	8.10 abc	12.1
TA	0.46 a	0.29 c	0.32 bc	0.30 c	0.37 b	5.9
Ratio	21.40 c	24.76 b	26.84 a	19.73 c	21.55 c	10.1
TC	10,323.08 a	2374.3 b	2758.9 b	1707.7 b	2066.7 b	8.5
VITC	183.8 b	213.2 ab	191.2 b	242.6 a	205.9 ab	8.2
TPC	677.2 c	802.2 ab	825.5 a	630.5 bc	695.5 abc	7.4
DPPH	1389.3 bc	1449.3 ab	1513.5 a	1315.3 c	1400.5 bc	2.8
ABTS	1489.6 bc	1633.9 ab	1642.0 a	1443.1 c	1520.8 abc	3.5
FRAP	7597.6 b	8059.4 a	8097.2 a	7127.41 c	7692.1 ab	2.2
Colors						
Luminosity	33.13 c	51.60 a	43.37 b	48.63 a	30.47 c	5.9
Chroma	31.37 b	50.57 a	44.33 a	45.83. a	22.40 c	8.2
Hue	30.63 c	72.33 a	50.77 b	71.87 a	28.80 c	6.9
Sensory						
Size	8.03 a	8.05 a	8.03 a	6.34 b	7.60 a	23.93
Shape	8.05 a	8.22 a	8.12 a	6.64 b	8.17 a	25.27
Color	8.74 ab	8.59 ab	8.30 b	8.27 b	9.05 a	20.33
Global acceptance	8.10 a	8.02 a	7.89 a	6.70 b	7.94 a	25.09

Fruit length (FL, mm), fruit diameter (FD, mm), pericarp thickness (PT, cm), [a] total soluble solids (SST, °Brix), titratable acidity level (TA, m/m citric acid), total carotenoids (carotenoids, mg beta carotene in 100g^{-1}), vitamin C content (VitC, mg 100 g^{-1}), total phenolic content (TPC, mg GAE g^{-1}), total antioxidant activity by Ferric reducing antioxidant power (FRAP, mg TEAC g^{-1}) and 2,2-diphenyl-1-picrylhydrazyl (DPPH●, mg TEAC g^{-1}), and antioxidant activity by a bts method (ABTS, mg TEAC g^{-1}). Size, shape, color and global acceptance luminosity (L), chromaticity (C), tonality angle (h), [b] Coefficient of variation (CV). Means followed by the same letter were not significantly different at Tukey test ($p > 0.05$).

By principal component analysis (PCA), the first two components explained 86.65% of the variation (PCA 1 and PCA 2 with 48.29 and 38.37%, respectively) (Figure 2a). PCA and hierarchical Ward clustered the genotypes into three groups (Figure 2). Group I consisted of the Moke genotype that presented high values for Chroma, Luminosity, Hue, PT, FD and VITC. Group II consisted of Kaiki and Akamu genotypes that obtained the highest values for Ratio, DPPH, FRAP, ABTS and TPC, while the Group III consisted of the Kalani and Kaolin genotypes. Kalani obtained the highest values for SS, FL, TC and TA, while Kaolin obtained intermediate values.

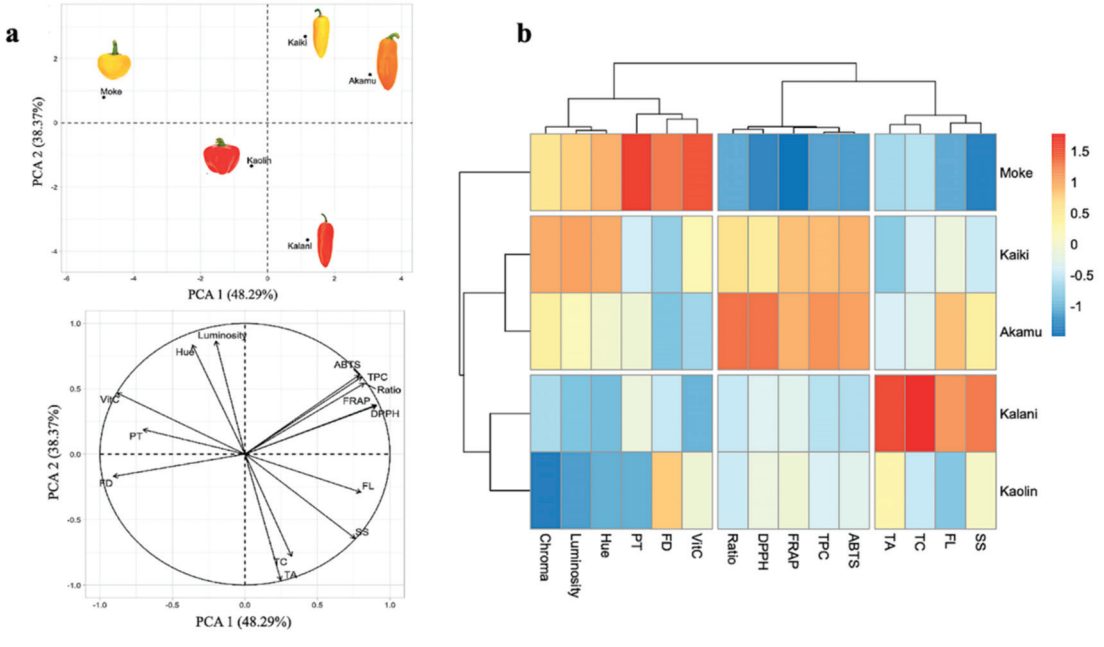

Figure 2. Principal component analysis (**a**) and Ward hierarchical grouping (**b**) of five mini chili genotypes for different morphological, biochemical and nutritional characteristics.Fruit length (FL, mm), fruit diameter (FD, mm), pericarp thickness (PT, cm), total soluble solids (SST, °Brix), titratable acidity level (TA, m/m citric acid), total carotenoids (carotenoids, mg beta carotene in 100 g^{-1}), vitamin C content (VitC, mg 100 g^{-1}), total phenolic content (TPC, mg GAE g^{-1}), total antioxidant activity by Ferric reducing antioxidant power (FRAP, mg TEAC g^{-1}) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•, mg TEAC g^{-1}), and antioxidant activity by a bts method (ABTS, mg TEAC g^{-1}). Size, shape, color and global acceptance luminosity (L), chromaticity (C), tonality angle (h), red/green ratio (a), yellow/blue ratio (b).

The fruit firmness analyses showed differences among mini sweet peppers genotypes (Figure 3). Moke and Kaolin obtained the highest peak, indicating that these fruits require a force of approximately 1.6 and 1.34 N, respectively, to perforate the exocarp. In turn, Kaiki fruits presented the less rigid exocarp, requiring 0.97 N of force applicable until rupture. These results were concordant when the firmness of the inner layers of the fruit (mesocarp) was analyzed, in which the genotypes Moke and Kaolin obtained greater resistance to perforation. It suggests that the Kaolin and Moke fruits have the most rigid peel, and the most elastic pulp, when compared to the other genotypes.

3.2. Sensory Evaluation

Regarding the profile of the evaluators, 58.1% and 41.9% were female and male, respectively. Most respondents were between 26 and 35 years old (43.9%), followed by respondents aged ≤25 years (31%), between 36 and 45 years (12.3%), >56 years (7.1%) and

between 46 and 55 years (5.7%). The evaluators were questioned about the frequency with which they consumed peppers. The answers were: 49.7% for eventually consume, 16.8% for monthly consume, 18.1% biweekly, 13.5% weekly and only 1.9% answered that they consume peppers every day.

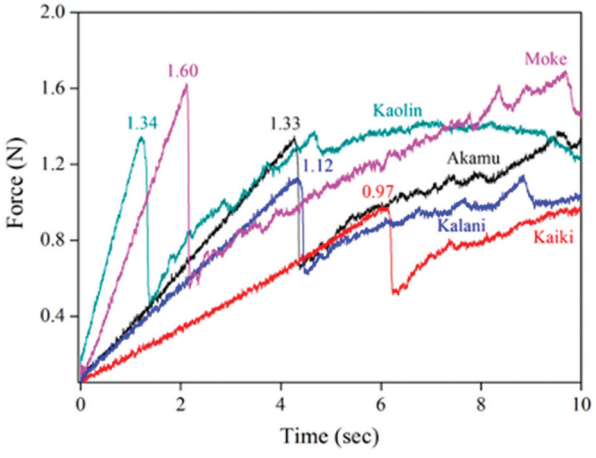

Figure 3. Texturogram of the five mini sweet peppers genotypes.

The lowest overall acceptance of consumers was to Moke fruits (Figure 4), while the highest acceptances for size and shape were observed for Kalani, Kaiki and Akamu. For color, Kalani and Kaolin who stood out positively. For global acceptance, higher scores were verified for Kalani, Kaiki, Kaolin and Akamu genotypes.

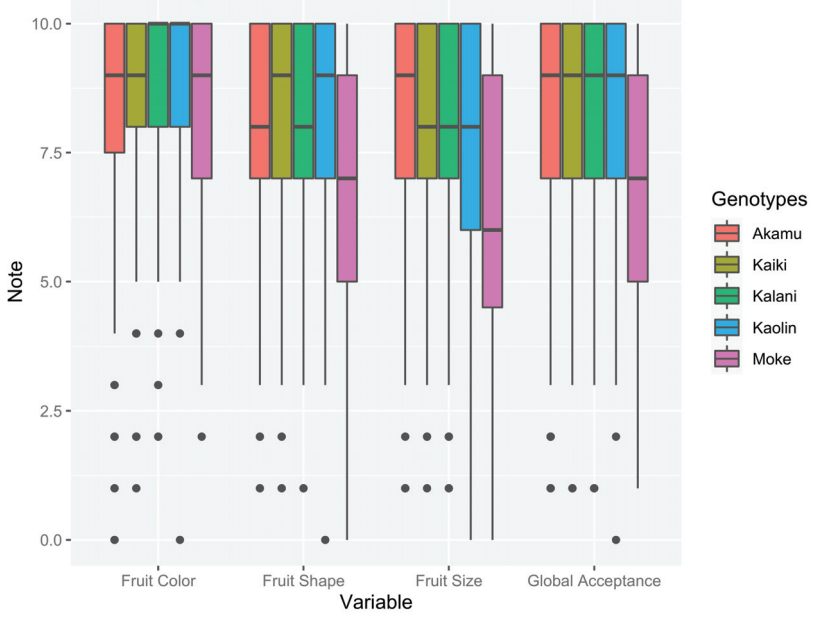

Figure 4. Boxplot analysis for the evaluators' scores for the sensory attributes of the five mini sweet pepper genotypes.

4. Discussion

The consumption of mini and baby vegetables has been growing over the years in Brazil, presenting an important appeal to consumers due to the ease of consumption and taste [8]. In addition, this type of vegetable provides a higher added value for rural producers, representing an excellent option for the diversification of production. The mini sweet peppers are new into the market with great potential, due mainly to the flavor, color, texture and shape of the fruits, being a great product to be consumed as an appetizer. Furthermore, its fruits have in their composition different substances related to improved health, such as antioxidant compounds and vitamin C, constantly related to the prevention of numerous diseases [19–21]. Therefore, this study envisioned to characterize different genotypes of mini sweet peppers regarding the physical-chemical and nutritional aspects and through sensory analysis to aware of the market potential of this vegetable.

Our study showed a wide variability among mini sweet pepper genotypes in physical, biochemical, nutritional and sensory aspects. Following [23], the commercialization of mini sweet peppers is extremely dependent on morphological variations, because the greatest acceptance comes precisely from the demand for fruit mix with different colors (yellow, orange, red and green). In this context, the use of the fruit mix can be considered an important strategy for the use of the nutritional potential of each of the genotypes evaluated.

The high SS values observed in mini sweet peppers (Kalani, Akamu and Kaolin) indicate higher natural sugar content, that is appreciated for fresh consumption as a healthy appetizer and for the industry aiming at fruit dehydration. Corrêa et al. [24], evaluating 16 sweet peppers hybrids, verified values ranging from 3.73 to 4.28 °Brix. Rinaldi et al. [25], evaluating field-grown peppers and hydroponics, obtained values from 4.90 to 7.40 °Brix. Kalani also stood out for total carotenoids, with values three to four times higher than other genotypes. Carotenoids are a group of phytochemicals responsible for the different colors of food. They have an important role for plant and animal health due to their nutritional traits and role in preventing degenerative diseases and protecting against oxidative stress [26,27]. In *C. annuum*, the main carotenoids are capsanthin, capsorubin, β-carotene, zeaxanthin, violaxanthin, lutein and antheraxanthin, which vary in concentration in different stages of fruit maturation [28,29].

The high vitamin C values found in the mini sweet peppers indicates their great nutritional potential, and it is an important trait for exploration in marketing. Mennella et al. [30], evaluating 15 genotypes of sweet peppers, found values ranging from 105.11 to 275 mg of ascorbic acid at 100 g^{-1} with an average of 167.47 mg of ascorbic acid at 100 g^{-1}. Nankar et al. [31], evaluating 180 accessions of *C. annuum*, found values ranging from 4.77 to 273.47 mg of ascorbic acid at 100 g^{-1} with an average of 115.59 mg of ascorbic acid at 100 g^{-1}. In the present study, the average vitamin C was 207.35 mg ascorbic acid at 100 g^{-1}, 3.5 times the recommended daily value for adults who consume 2000 calories [32].

Sweet pepper fruits are also rich in phenolic compounds, which are beneficial to health due to their ability to eliminate free radicals in biological systems in vitro and in vivo [26,33]. This fact was corroborated by the high correlation between CPT and methods that determine antioxidant capacity in vitro (DPPH, FRAP e ABTS), where Kaiki and Akamu fruits presented the may values. Antioxidants are known as cellular protectors for the fight of free radicals in the body, not allowing the oxidation process. Series of free radical-initiated reactions cause membrane data, disrupting metabolic pathways, thereby increasing DNA mutations and changes in platelets and other functions [34].

The greater firmness of the fruits may be related to the shape of the most found fruit, requiring greater strength for the disruption of the exocarp and mesocarp. However, this shape of the fruit presented lower acceptance among the interviewees. Vision is an important parameter in sensory analysis, because it is the first impressions of the products. Despite the small size of these vegetables, the market for mini sweet peppers has gradually increased and increasingly conquers the consumer. At the same time, the development of cultivars that present special characteristics is required by the industry, not only in relation to fruit morphology, such as attractive nutritional size and nutritional format,

but also by selecting cultivars that unite all the benefits that the species can provide. The appearance of a product is one of the most important factors evaluated by the consumer at the time of purchase and; therefore, a mix of fruits of different colors and elongated shape of mini sweet peppers can be considered an important strategy for commercialization, associating with the nutritional quality of this group of vegetables. Summing up, our results provides excellent information for research and industry about the characteristics of the mini sweet peppers. These data are important to provide pathways and assist in the creation of strategies for both improvement and trade, to the development of more nutritious and attractive vegetables. Therefore, it also can be applied to several other classes of mini vegetables.

5. Conclusions

A wide variability was observed among genotypes for physical-chemical and nutritional traits. Based on sensory analysis, Kalani, Kaiki, Kaolin and Akamu obtained greater global acceptance. The data that have been presented here are just the beginning of a characterization about the mini sweet pepper genotypes. These mini vegetables have greater potential to be explored and accordingly several other analyses are necessary to better understand and get information about their characteristics and qualities and thus design new perspectives and strategies for improving and increasing the consumer market. The genotypes can be considered an important marketing strategy of mini sweet peppers trade, associating different shapes, colors and nutritional quality. Whereas several public policies and innovations in the agricultural sector have been carried out in order to encourage the consumption of vegetables, this information can contribute to the development programs of new cultivars, with focused efforts on mini vegetables that are not only attractive by shape and color, but also associate with the excellent nutritional characteristics.

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Review

Phenotyping Brown Rot Susceptibility in Stone Fruit: A Literature Review with Emphasis on Peach

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Abstract: Plant disease phenotyping methodologies can vary considerably among testers and often suffer from shortcomings in their procedures and applications. This has been an important challenge in resistance breeding to brown rot, one of the most severe pre-and postharvest stone fruit diseases caused by *Monilinia* spp. Literature about methodologies for evaluating stone fruit susceptibility to brown rot is abundant but displays significant variations across the described approaches, limiting the ability to compare results from different studies. This is despite the fact that authors largely agree on the main factors influencing brown rot development, such as *Monilinia* inocula, environmental conditions, cultivars, fruit stage, and management practices. The present review first discusses ways to control or at least account for major factors affecting brown rot phenotyping studies. The second section describes in detail the different steps of fruit infection assays, comparing different protocols available in the literature with the objective of highlighting best practices and further improvement of phenotyping for brown rot susceptibility. Finally, experimental results from multi-year evaluation trials are also reported, highlighting year-to-year variability and exploring correlations of evaluation outcomes among years and assay types, suggesting that choice of phenotyping methodology must be carefully considered in breeding programs.

Keywords: brown rot; inoculum application; *Monilinia*; phenotyping; phenotypic instability; stone fruit

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1. Introduction

Brown rot (BR) caused by *Monilinia* spp. is one of the most destructive diseases in commercial stone fruit orchards worldwide. *M. fructicola* (G. Winter) honey, *M. laxa* (Aderh and Ruhland) honey, and *M. fructigena* (Aderh. and Ruhland) honey are the main species causing fruit infections [1]. These fungi incite losses by infecting blossoms, flowers, and fruit during the preharvest, harvest, and postharvest periods [2]. Postharvest losses can be particularly severe, especially when conditions are favorable for disease development; in some cases, 80–85% of a crop may be lost [2,3]. When weather conditions are unfavorable, infections may remain latent until conditions become favorable for disease expression, at which point fruit rot ensues [4].

Currently, cultural practices and frequent fungicide applications are the main management measures to control BR in the field, although emerging *Monilinia* isolates resistant to fungicides have been reported [5,6]. Therefore, developing and assessing cultivars with resistance traits against BR has been the primary goal of several breeding programs.

Classic breeding approaches are time-consuming due to lengthy procedures for evaluating resistance on field-grown segregating progenies. Therefore, an important objective is to develop new tools to screen seedlings with enhanced BR resistance. Marker-assisted selection (MAS) is a valuable strategy for this purpose, as it allows the early selection of seedlings bearing favorable alleles at marker loci associated with genomic regions that

control the trait of interest. In stone fruit, the mapping of quantitative trait loci (QTL) on populations derived from bi-parental crosses is presently applied [7–9]. However, genetic analyses require accurate phenotypic data for the estimation of genotype-associated variation of the trait.

BR resistance is a complex trait requiring robust, easy to apply, inexpensive and effective phenotyping methods. Many stone fruit breeders have developed protocols aiming at BR susceptibility evaluation on fruit [10–14]. Some are applied in the field, others in controlled conditions (laboratory); some are easy to use, whereas others involve laborious procedures. However, a complete understanding of the process that contributes to effective disease phenotyping is crucial for results to be reliable and repeatable. Protocols are highly dependent on adequately performing different steps. In addition, other factors influence the development of BR and also directly affect the phenotyping process. No comprehensive review is available on phenotyping methodologies for brown rot susceptibility in stone fruit to this extent.

Therefore, this review focuses on essential phenotyping protocols and procedures applied in breeding programs and cultivar evaluations for BR susceptibility in stone fruits. The objectives were to (i) summarize essential factors for BR development and phenotyping, (ii) review the protocols applied in the field and laboratory for artificial BR infection, and (iii) discuss consequences and instability in phenotyping, also in light of recent unpublished experimental results from our group.

2. Factors Influencing Brown Rot Development

The critical life stages of *Monilinia* spp., such as primary inoculum availability, host infection and colonization, and secondary inoculum, are the essential prerequisites for the development of BR infection. Multiple factors influence the completion of these life stages, and their knowledge is critical to developing optimized phenotyping protocols.

Principally, the brown rot life cycle includes different stages [1]: blossom blight and twig canker at early spring, brown rot at late spring and summer, latent infections, and overwintered inoculum in the form of mummified fruit on trees or orchard ground.

Monilinia spp. overwinters and produces primary inoculum from two sources: mycelia in the fruit mummies, fruit peduncles, cankers on twigs and branches, leaf scars, and buds that sporulate under favorable condition; and stromata that produce ascospores in the spring [1,15–19]. However, mummies hanging on trees appeared to be a more viable and effective source of primary inoculum than ground mummies [20].

Secondary inoculum can emerge from any infected tissue in which the moisture content is sufficient for sporulation [1]; however, non-abscised (aborted) fruit on trees and thinned fruit on the orchard floor appeared to be critical sources in certain production regions [21,22].

Some authors remark the importance of quiescent infections on developing or ripening fruit that may become active when fruit mature before or after harvest [23]. Latent infection can be particularly relevant postharvest [24]. Molecular techniques have been developed for detecting latent infections in stone fruit [25,26]. Latent infection is critical for postharvest BR epidemiology, although it is less discussed at the breeding level.

2.1. Environment

Environment plays an essential role in disease development [27]. Variables such as temperature, photoperiod (light), humidity, and leaf wetness modulate canopy environment and influence fruit growth and quality [28], as well as BR development. For *Monilinia* spp., the most critical environmental factors seem to be temperature and humidity. Under favorable conditions, the process of *Monilinia* infection starts with the conidium germination on the fruit surface, followed by elongation of the germ tube and formation of appressoria to penetrate the epidermis [29] or to enter through natural openings and wounds [30]. Under adverse conditions, primary infections can remain latent in blossoms and immature fruits [23,31].

Temperature and humidity are primary factors to be considered in the *Monilinia* spp. life cycle. The optimum temperature for mycelial development and sporulation was about 25 °C for all BR fungi [1]. However, for most *Monilinia* spp., the optimum temperature for mycelial growth ranges from 15 to 20 °C, and only *M. laxa* requires 25 °C [32]. Regarding *M. fructicola* germination, the best temperature range has been reported at 15–25 °C or 21–27 °C, depending on the study [33,34]. More recently, analyzing the influence of temperature on fruit infection, Biggs and Northover [35] suggested that optimum temperature for cherry and peach BR infection by *M. fructicola* ranged from 20 to 22.5 °C and 22.5 to 25 °C, respectively.

Bernat et al. [36] modeled and compared the effects of temperature on brown rot, mycelia development, and sporulation on peaches and nectarines for *M. fructicola* and *M. laxa*. They showed a better adaptation of *M. fructicola* and *M. laxa* to high and low temperatures, respectively. Notably, the capacity of *M. fructicola* and *M. laxa* to infect fruit seems to be maintained across an extensive temperature range, between 0 and 30 °C [36]. In addition, the two species significantly differ in infection and colonization speed, whereby *M. fructicola* is more aggressive, causing larger fruit lesions and having shorter periods of both incubation and latency [37]. However, the risk of *Monilinia* infection is significantly reduced at low temperatures [38].

Several reasons can explain discrepancies among studies: relative humidity and/or temperature-by-humidity interactions; the different optimal temperatures required for fungal functions, such as germination, mycelial growth, and sporulation; variations in temperature requirements putatively existing between geographic isolates of *M. fructicola*: e.g., isolates from blossoms, which develop during cool springs, grow at lower temperatures than those developing on fruit [39]. However, temperatures deviating from the optimum mainly cause a delay of germination but have a limited effect on the final infection success [40].

Wetness or relative humidity (RH) influence the initiation and development of BR in many inter-related ways. In sweet cherry, BR incidence by *M. fructicola* doubled when wetness duration increased from 9 to 12 h and doubled again with further increase in wetness duration [35]. Similar results were also reported on peach, where a linear increase in disease incidence was observed over the same conditions. Likewise, blossom infections by *M. laxa* were significantly influenced by both temperature and duration of post-inoculation wetness [41]. The degree and course of wetness also influenced the success of penetration of nectarine surface and disease incidence [42]. In the same way, the penetration of peach blossoms by *M. fructicola* was greatly influenced by relative humidity [33]. In a saturated atmosphere, access occurred through any of the floral parts, except sepals, but at a relative humidity of 80% or lower, infection was only observed through stigmas [4]. A combination of those two factors determines the delay before infection and the likelihood of success. Under dry conditions at 15 °C, up to 40% of cherry blossoms were infected, while infections at different temperatures (5, 10, and 20 °C) were less frequent. In contrast, under 24 h post-inoculation wetness, up to 70–90% of blossoms were infected at each temperature tested [42].

Furthermore, wind is another crucial factor, as it could modify relative humidity and conidium dispersion through air turbulence [43], playing an essential role in disease spread.

Finally, rain is another significant factor in BR development, assisting in dispersing and spreading inocula and providing ideal relative humidity. Further information on the epidemiology of *Monilinia* spp. has been reviewed by Holb [44] and Rungjindamai et al. [45].

2.2. Cultivars

Despite being most relevant for breeding, qualitative sources of BR resistance have not been found in peach and other stone fruit. Some studies have identified accessions with partial quantitative resistance (often erroneously defined as highly tolerant), in which

infection remains latent and/or a limited number of fruits per tree develop symptoms; however, available commercial cultivars are all relatively highly susceptible to BR. Such high susceptibility acts as a further contributor to BR development since infected fruit serve as a continuous inoculum source along the season. In peach, the Brazilian cultivar Bolinha is known to display the highest levels of BR partial resistance in terms of reduced rate of lesion development, sporulation per unit area, and, particularly, disease incidence [46,47]. This cultivar has been used as a BR resistance donor in conventional breeding for developing canning and low-chill peaches despite its poor fruit size and quality, high susceptibility to enzymatic browning, and high incidence of preharvest fruit drop. Besides the increased compactness of epidermal and sub-epidermal cells, the high fuzz and thick cuticle, Bolinha fruit contain a high amount of phenolic compounds compared to other BR-susceptible cultivars [48,49]. The case of 'Bolinha' demonstrates the challenge of breeding for BR resistance, as traits associated with fruit resistance may conflict with commercial requirements; however, among the primary objectives of some breeding programs, resistance against BR takes precedence.

In the peach breeding program at the University of Milan, Italy (started at the University of Bologna), an F1 population from a cross between 'Contender' × 'Elegant Lady' [50] resulted in a higher BR partial resistance level compared to the donor 'Contender' [8].

At UC Davis and USDA joint breading program, improved levels of BR partial resistance in some peach cultivars and advanced selections were reported. A progeny was generated by crossing the moderately resistant cultivar Dr. Davis with an introgression line ('F8,1–42') resistant to BR, originated from an almond × peach interspecific cross [7].

Furthermore, at the Clemson University peach breeding program, some degree of resistance has been reported in materials other than 'Bolinha' and interspecific hybrids (almond × peach). An advanced selection from the North Carolina State University peach breeding program 'NC97-45' ('Contender'; descendant) [51] was reported as more resistant to BR than parents [52], which supports the findings of Pacheco et al. [8] on 'Contender' as a source of partial resistance to BR.

In another program, the progeny from 'Texas' (almond) and 'Earlygold' (peach) backcross (BC1) showed a wide range of severity and incidence of BR infection in wounded and non-wounded fruit [9]. Moreover, Nicotra et al. [53] have reported 11 advanced apricot selections and cultivars with BR-resistant traits. However, studies in many cherry cultivars failed to find promising accession with fruit resistance to BR [54–56]. In contrast to the low level of skin tolerance often found in peach, plum cultivars showed low [12] or no BR infection [57] in inoculated intact fruits. Thus, the outcome of inoculation of intact fruit surface (skin) seems unsuitable for artificially classifying plum fruit as BR tolerant since they are still sensitive in a natural condition or when fruit are wounded.

2.3. Fruit Stage

Fruit susceptibility to BR varies along with the phenological growth and development stage. Several studies have investigated these variations by evaluating infection probability at different fruit stages [58].

In peach, fruit development is divided into four stages (S1 to S4), all highly susceptible to *Monilinia* spp. except for S2 (pit hardening) [59]. The early fruit stage-related susceptibility to BR on stone fruit has been previously reported [58,60].

The first stage (S1) starts after ovule fertilization or petal fall and ends at the beginning of stone lignification. The fruit is photosynthetically active at this stage, displaying intense transpiration activity and showing the highest nutrient content [61], resulting in increased susceptibility to BR, probably due to the stomata activity, providing an entry point to the pathogen [62].

The second stage (S2, pit hardening) is the most resistant to *Monilinia* spp. infection [58,63,64]; this stage is characterized by the accumulation of secondary metabolites, such as catechin, epicatechin, and phenolic compounds, associated with the lignification of the endocarp. In artificially wounded fruit, the temporary absence of susceptibility in S2 seems to be mainly associated with

the biosynthesis of specific biochemical compounds rather than a higher mechanical resistance [58]. Contrary to other studies, even the pit hardening stage has been observed to be susceptible to BR infections, which remain latent until the ripening stages [59,65].

During the third stage (S3), characterized by a high rate of cell expansion and ending at fruit physiological maturity, fruit become increasingly susceptible to pathogens, including BR. At fruit maturity (S4), BR susceptibility reaches its peak starting approximately two weeks before full ripening [35,63]. Similar patterns were previously reported for apricot and peach [58,64]. The progressive decrease in resistance-compounds concentration due to fruit growth and/or structural changes affecting surface integrity would seem the most plausible hypotheses for the increased susceptibility observed at these stages [48].

Also, in cherry, the susceptibility to *M. fructicola* fluctuates with the stage of fruit development [66]: young developing cherries become increasingly susceptible to infection, then they turn to be less susceptible at pit hardening and finally again become gradually more susceptible until harvest [56]. Moreover, the susceptibility to *M. laxa* under field conditions significantly increases with fruit maturity [56].

2.4. Cultural Practices and Orchard Management

Commonly applied practices in a stone fruit orchard, including crop load management, irrigation, fertilization, pruning, and canopy architecture, have a major impact on *Monilinia* spp. development [67]. Besides fungicide application, pruning blighted twigs and removal of mummified fruit are considered the most effective control measures against BR. Cultural practices can impact the inoculum source directly via microclimate modulation such as irrigation, pruning, fertilization, and indirectly via fruit thinning [68].

Mercier et al. [69] studied the combined effects of irrigation regime and pruning system. The lowest BR incidence occurred under a combination of water deprivation (about 30% of the fully irrigated treatment) and 'long' pruning (i.e., dormant plus summer interventions for the removal of epicormic shoots and young, vigorous sprouts, without trimming) in comparison with full irrigation and 'short' pruning (i.e., dormant plus summer interventions of shoot trimming). Similarly, training system and pruning (shapes with a dominant central leader) seemed to reduce brown rot incidence compared to conventional system, e.g., 'vase' systems [70]. This effect could be due to improved light penetration and reduced relative humidity in the less dense canopies that negatively affected fungal germination and sporulation.

Gilbert et al. [71] have primarily studied the complex interplay between cultural practices, fruit growth, and BR infection risks. They showed that irrigation and fruit thinning affect fruit growth and the appearance of microcracks on the fruit surface. Frequent and high levels of irrigation on 'Zéphir' nectarine strongly increased the density of cuticular cracks compared to water-restricted trees receiving two- to three- times less water per day. Furthermore, low crop loads dramatically increased both fruit size and the incidence of cuticular microcracks, leading to increasing BR susceptibility.

Nevertheless, management of crop load concerning fruit BR susceptibility seems difficult to be optimized. Bellingeri et al. [72] reported opposite effects on trees subjected to different thinning treatments, with the highest BR infection observed in moderately thinned compared to intense or unthinned trees. This could be explained by a complex interaction between the probability of infection by contact (which tends to decrease along with fruit density) and cuticle cracking (which tends to increase in faster-growing fruit) (see Section 2.5).

Fertilization also seems to play a role in BR susceptibility. For example, peach trees subjected to a high level of compost exhibited a significant increase in *M. fructicola* incidence. Other studies investigated fertilization with calcium [73], zinc [74], and boron [75], reporting an enhancement of fruit quality and lowering of BR susceptibility. The effect of fertilization could result in a modification of tree growth, affecting canopy microclimate or increased fruit nitrogen content [76]. However, no clear correlation between seasonal changes of peach nutrient content and susceptibility to *M. laxa* was found [61].

2.5. Fruit Characteristics

Monilinia spp. can mainly enter the fruit via two ways, either by actively penetrating the fruit surface or through natural openings such as stomata or microcracks (Figure 1) [30,48,77–79]. However, *Monilinia* is also able to penetrate fruit skin directly without the need for wounds or natural openings, employing degrading enzymes and colonizing plant tissue similarly to other necrotrophic fungi [79–83].

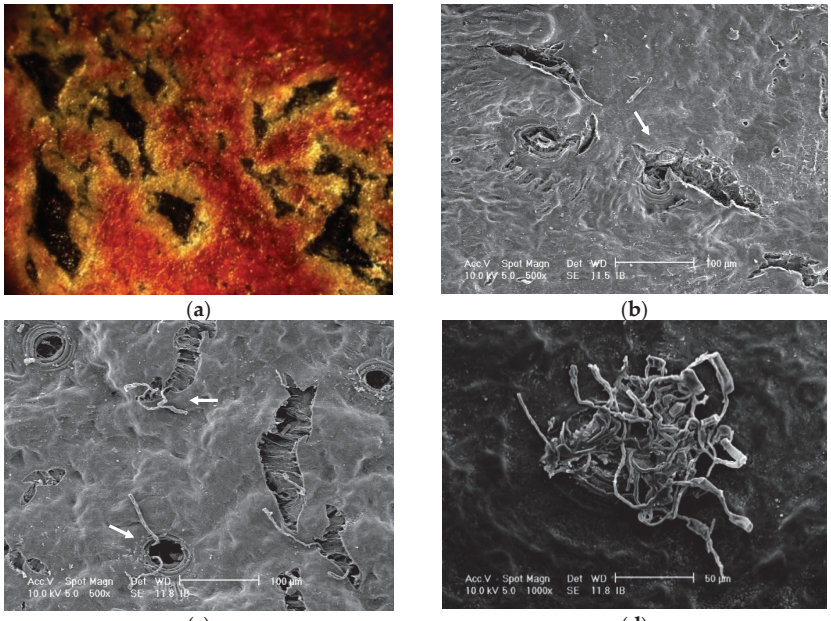

Figure 1. (**a**) Surface of the nectarine fruit 'Zéphir' at maturity with a dense network of microcracks under a stereomicroscope. Cracks were stained dark blue by applying toluidine blue at 0.1%. (**b**) Scanning electron microscopy image showing microcracks originating from a lenticel, presumably derived from a stoma (arrow) on the fruit peel of nectarine 'C222' selection. (**c**) Scanning Electron Microscopy image showing spores of *M. laxa* germinating (arrows) in the microcracks of mature nectarine fruit of cultivar Magic. (**d**) Scanning Electron Microscopy showing the development of *M. laxa* mycelia in a lenticel on the fruit peel of 'C222' selection.

Besides chemical factors such as nutrients and volatiles, fruit surface characteristics such as hydrophobicity and topography are common appressorial inducers for many fungi. In nectarine fruit, the formation of *M. fructicola* appressoria at the S2 stage and their absence at the S3 stage seem to be associated with the respective high and low peel hydrophobicity [29].

Although *Monilinia* is a necrotrophic fungus that can infect fruit via direct penetration, fruit cracks are well-known to be the preferential entry ports [84,85]. Different fruit characteristics can be accounted for reducing susceptibility to BR, which most of these defense barriers, either mechanical or biochemical, are related to the epidermis [12,46,48]. Considering the active penetration of the fungi, the composition of the different epidermis layers and the mechanical traits linked to surface integrity seem to be the main characteristics to be explored in addition to active biochemical defense mechanisms.

The plant cuticle is the first protective barrier to biotic stresses, as it contains antimicrobial compounds involved in plant-pathogen interactions. However, until recently, few studies have explored the cuticle of *Prunus* fruit. Oliveira Lino et al. [60] studied the cuticu-

lar wax composition of three nectarine cultivars and its change during fruit development in correspondence to skin conductance and susceptibility to *M. laxa.* Cuticular waxes greatly varied both quantitatively and qualitatively throughout fruit growth. The high skin conductance in the early stages was attributed to the high density of functional stomata in young fruit and the absence of the wax layer not yet formed. Moreover, this absence might have also facilitated direct infection by *M. laxa* at the early stages. The variation of cuticular wax deposition may also explain their contribution to BR resistance at pit hardening and, conversely, the susceptibility of mature fruit (showing a higher level of alkane waxes, which could favor the fungus growth).

Skin cracks are an essential factor affecting the integrity of fruit surface integrity. The link between cracking and BR incidence suggests that fruit resistance factors provided by the epidermis are, of course, no longer influential when the cuticle loses its integrity [71]. Cuticular cracks are assumed to occur when the elastic limit of the cuticle is exceeded as a consequence of high internal pressure, especially during rapid fruit expansion [86,87]. Certain cultural practices mainly promote a fast-growing phase (see Section 2.4). Microscopic observations of fruit surface in three nectarines ('Zéphir', 'Magic', and 'C222') confirmed the formation of a dense network of microcracks in mature fruits and preferential spore germination inside the cracks (Figure 1). These observations suggest that BR resistance factors targeted in breeding programs should explore a combination of these two traits: low susceptibility to cracking and enhanced content of antifungal compounds.

3. Protocols for BR Susceptibility Evaluation

Some stone fruit breeders and scientists have developed protocols for BR susceptibility evaluation to be applied either in the field or controlled environments (laboratory); some are easy to use, whereas others involve laborious procedures. The goal commonly sought is a robust, fast, and low-cost protocol enabling the screening of a large number of progenies. This section reviews BR resistance phenotyping protocols used to evaluate artificial infection in stone fruit, focusing on cultivar evaluation and breeding programs.

Among the several prerequisites, assessed fruit should not receive fungicide treatments after flowering [13,88] since fungicide residues could bias phenotyping results. Selected fruit should also be unblemished, uniform in size and maturity [47] since variations in the degree of ripeness and/or the presence of wounds or cracks could mislead conclusions about fruit susceptibility. Criteria and methods for establishing the degree of fruit maturity often vary across studies, ranging from visual assessment to the measurement of firmness, color, and/or soluble solids content (SSC) [10,11]. The use of the index of absorbance difference (I_{AD}) measured by a portable DA-Meter (TR Turoni, Forli, Italy) seems a reasonable and objective approach to standardize peach maturity evaluation [11,14,89,90]. In addition, a stereomicroscope was used to examine fruit surfaces with the aim of discarding injured fruit before inoculation [46]. However, this procedure is difficult to implement as a routine check.

3.1. Fruit Preparations before Inoculation

In laboratory assessments, fruit are carefully handpicked and usually subjected to preparations before inoculation [91]. Primarily, damaged and field-infected fruit are excluded [91] without considering possible latent infections coming from the field that have not yet been activated. Dissipating field heat or precooling of fruit is the first care to slow down biological activities. To this end, different fruit temperatures and treatment durations, for example, storage at 0, 0.5, and 4 °C for few days up to few weeks, have been tested [7,11,57] until the day of assessment. However, prolonged storage is not recommended since low temperatures may interfere with critical physiological properties and modify fruit susceptibility. Storing fruit for short periods gives more flexibility to organize inoculation. For example, Gradziel et al. [46] kept fruit at 22 °C for 12 h to homogenize the batches harvested on different days and simulated the practice of fruit storage in the postharvest and commercialization period.

Postharvest disinfection of fresh fruit is considered an essential step before handling [92]. Similarly, this practice has been employed in screening stone fruit for BR susceptibility before inoculation with *Monilinia* spp. to eliminate field contaminations or competing organisms that may interfere during artificial infection. Fruits were surface-sterilized by bleach at 10% or 8%, with different concentrations of sodium hypochlorite (NaOCl) [7,36,53,54,90], calcium hypochlorite [93], or less concentrated chlorine solutions ranging from 0.5% to 2% [4,10,37,53,56,64,94]. In addition, ethyl alcohol has been used as a surface sterilizer, mainly at 70% concentration, before or after disinfecting with chlorine compounds [4,37,54]. However, no consensus method for disinfecting fruit before inoculation emerged from these protocols, as different concentrations and combinations of hypochlorite, ethyl alcohol and timing have been used. However, in all methods, the process ends up carefully rinsing fruit in water to remove the disinfectants, followed by air drying. Overall, the treatments above might be considered as disruptive of the fruit surface and, putatively, a modification of its susceptibility to infection. This was the reason behind the use of only water for fruit cleaning in some studies [50,55].

Baró-Montel et al. [11] have thoroughly investigated the effect of different types and concentrations of disinfectants on wounded and non-wounded fruit before inoculation. They reported a lower disease severity in disinfected wounded fruit. However, in non-wounded fruit, a significant increase in disease severity was reported when the most aggressive (10% NaClO) disinfectant treatment was applied. Finally, they also observed a rise in BR incidence after dipping the fruit in tap water without a disinfectant, suggesting that water could promote pathogen growth and facilitate the infection process.

The use of a water bath (recommended as a technique to reduce postharvest infections) deserves further attention. Spadoni et al. [95] have shown a stimulating effect on the germ tube of *M. fructicola* conidia on the fruit surface immediately after heat treatment at 60 °C for 60 sec. Volatile organic compounds emitted from heat-treated peaches have been putatively implicated in the stimulation of conidium germination and the increased BR incidence when inoculation occurred immediately after bathing.

We further investigated the effect of water bath on BR infection on three nectarine cultivars. Twenty fruit per accession at commercial maturity were chosen and subjected to soaking in 55 °C hot water for 45 s. Then, fruit were air-dried at room temperature, followed by inoculating with droplet at concentration 10^5 conidia mL^{-1} of *M. laxa*. In this experiment, a significant increase in BR infection probability was observed on water bathed fruit compared to unbathed ones (Figure 2). The effect of water bath on surface compounds for the cultivar Zéphir was also investigated. However, we detected no significant differences between the two treatments on the contents of triterpenoids or terpenoid derivatives in the fruit peel (data not shown). Even though these surface compounds were not affected, the water bath might influence other compounds such as proteins and water-soluble metabolites involved in the fruit-fungus interaction trade-off pathway. A similar increase in BR incidence has been reported for peach and nectarine [38] and nectarine 'Red Jim' fruit [24] when subjected to water dumping followed by incubating at 20 °C 65–100% RH.

Even though fruit disinfection is an important operation in postharvest trials to avoid secondary infections, our results recommend utmost precautions before subjecting fruit to the water bath since this procedure seemed to increase the susceptibility of nectarine fruit to BR and may activate latent infections in postharvest handling.

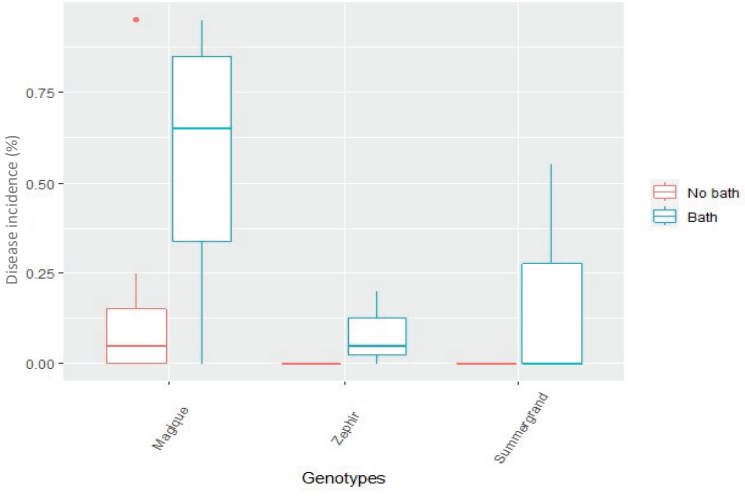

Figure 2. The effect of water bath on the brown rot disease incidence of three nectarine cultivars Magique, Zéphir, and Summergrand. Fruit were immersed in hot water (55 °C) for 45 s. The air-dried fruit at room temperature were inoculated with a 10 µL drop of *M. laxa* suspension at concentration 10^5 conidia mL^{-1} (p-value < 0.005).

3.2. Strain Conservation and Inoculum Production

Monilinia spp. culture could be maintained for long-term storage on different media such as potato dextrose agar (PDA) at 5 °C [96] or 4 °C [10], and 2% Malt extract agar at 2 °C in darkness [97]. There are other methods for storing fungi; for example, in our lab, we maintained *Monilinia* spp. spores in an aliquot of 20% glycerol with potassium dihydrogen phosphate buffer at −20 or −80 °C.

Before running any experiments, suitable quality inoculum should be prepared. Therefore, *Monilinia* spp. cultures are activated on nutrient media at optimum temperatures (25 °C). Inoculum preparation from a single-spore isolate allows using the same isolate throughout the experiment. However, some authors used the isolated *Monilinia* spp. directly from seasonal infected stone fruit: in this case, series of subcultures are needed to purify the inoculum from contaminants.

Moreover, the assessment of pathogenicity and virulence among *Monilinia* species revealed a significant variability even among isolates of each species [65,94]. Thus, it is recommended to check the stability of pathogenicity before running experiments. According to Koch's postulates, such a practice can be performed by infecting intact fruit (e.g., peach) [10]. In our lab, working on *M. laxa* and *M. fructicola*, we observed reduced growth competence of *Monilinia* spp. on V8 juice agar (V8A) after several subcultures. Therefore, we periodically regenerated new cultures from aliquots stored at −20 °C or isolating from actively infected fresh fruit (Figure 3). This process was repeated every three months to maintain maximum growth speed.

Screening large progenies for BR susceptibility requires a tremendous amount of inoculum to be prepared weekly to achieve an identical concentration of viable conidia throughout the experiment. The media composition may impact the rapidity of growth and sporulation, the number of spores produced, and viability.

PDA and V8A are the most common media used for inoculum production for Monilinia; other less frequent media include peach or tomato juice agar and glucose-asparagine-yeast extracts (Table 1). Producing the inoculum directly on fruit is a valid and viable option (Figure 3), with the precaution of previous disinfection with alcohol. The use of canned fruit is also reported [13]. Phillips [98] reported that spores produced on PDA were less

aggressive and smaller in size than those cultivated on peach and nectarine fruit. Hence, a culture media as V8A may be preferred for high quality and amount of sporulation.

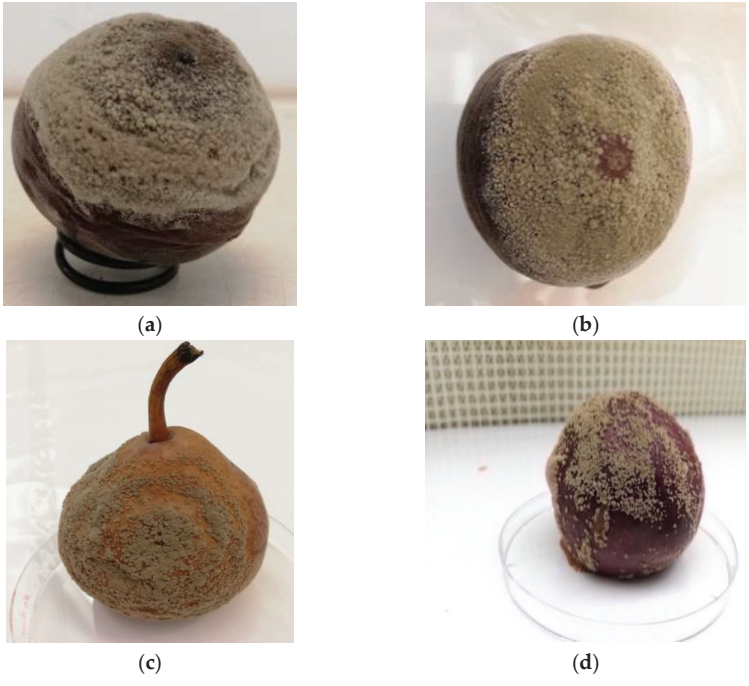

Figure 3. *M. fructicola* inoculum production and activation on peach (**a**) and *M. laxa* inoculum production and activation on peach, pear, and plum (**b**, **c**, and **d**, respectively) at 7 days post-inoculation. The inoculated fruit, with 10 µL at 10^5 conidia mL^{-1} suspension concentration for each species, were incubated in a culture chamber at 24/18 °C and 16/8 h light/dark photoperiods, in clear plastic boxes with maximized relative humidity.

For inoculum production, culture plates (i.e., V8A or PDA) are incubated between 20 and 25 °C under different photoperiods. Light is regarded as an essential promoter for conidium production. Authors have tried to produce inoculum under different photoperiods: 12 h light/dark or 16 h light/8 h dark; also, continuous light or dark were tested (Table 1), even though *M. fructicola* appeared to require shorter photoperiods than *M. laxa* to effectively sporulate [36], a 16 h light/8 h dark photoperiod is based on our experience advisable to promote sporulation for both.

The time to promote sporulation of *Monilinia* Petri dish cultures is another phase that differs in literature. Depending on the type of medium and incubation condition, authors have used 5 to 14 days old cultures for inocula [10,36,58,99]. Though this period is critical, it should not exceed 14 days, especially for sporulation quality.

Table 1. Phenotyping protocols for evaluating brown rot disease susceptibility in stone fruit.

Fruit Species	*Monilinia* spp.	Maturity Determination	Wounded or Unwounded (Intact)	Production of Inoculum	Mode of Inoculation	Inoculum Concentration (conidia/mL)	Incubation Condition	Assessment Time	Disease Assessment	Reference
Peach	*M. fructicola*	Fruit color determinations by spectrophotometer	Unwounded, wounded	V8A	Drop 10 µL	2.5×10^4	Humidified plastic containers at room temperature	3 days	Disease incidence, disease severity (lesion diameter)	[7]
Peach	*M. fructicola*	Mature (firm ripe) and mature green	Unwounded, wounded	PDA	Drop 10 µL and a 5-mm mycelial disk	2×10^5	23–25 °C/90% RH in dark	(24, 48 and 73, 96 h), rote diameter (48, 72, and 96 h) and sporulation 7 days	Disease incidence, disease severity (rot diameter), sporulation amount	[47]
Peach	*M. fructicola*	Commercial maturity	Unwounded	PDA	Drop 10 µL	2×10^4	22–25 °C/95% RH, in dark	3 days	Disease incidence	[46]
Peach, Nectarine, Plum	*M. fructicola*	Commercial maturity	Unwounded, wounded	PDA + acidified lactic acid	Drop 20 µL	1×10^6, 10^5, 10^4, 10^3, 10^2	20 °C/95% RH in plastic cardboard boxes	5 to 7 days	Disease incident and severity (lesion diameter)	[57]
Peach, Nectarine	*M. fructicola*	Maturity classes based on (I_{AD})	Unwounded, wounded	PDA supplemented with tomato pulp	Drop	2.5×10^4	20 °C and 85% RH storage boxes	3 and 5 days	Brown rot incidence (%), lesion diameter	[11]
Peach, Nectarine	*M. fructicola*, *M. laxa*	Commercial maturity	Wounded	PDA	Drop 15 µL	1×10^4	0, 4, 10, 15, 20, 25, 30, 33 °C with ±1 °C/85% RH, dark or 12-h light photoperiod	12 h for *M. fructicola* and 5–7 days for *M. laxa*	Lesion diameter, presence or absence of sporodochia	[36]
Peach, Apricot, Sweet cherry, Plum	*M. fructicola*, *M. laxa*	Commercial maturity	Wounded	V8A	Drop 30 µL	1×10^5	22 °C/high RH, in containers	6 days	Disease severity (rot diameter)	[39]
Peach, Nectarine	*M. fructicola*, *M. laxa*, *M. fructigena*	NA	Wounded	PDA	Drop 25 µL	1×10^4	22 ± 2 °C/light and in humidity chambers lined with a moist paper	7 days	% brown rot incidence, lesion diameter, sporulation, spore germination, mycelium length	[37]
Peach, Nectarine, Apricot, Plum	*M. fructicola*, *M. laxa*	Commercial maturity, immature fruit	Unwounded, wounded	V8A, PDA	Filter paper disks soaked in suspension, drop 10 µL	1×10^4	22–25 °C/(90–100%) in plastic boxes lined with a damp paper towel and the lids closed	7 days	Pathogenicity and disease incidence	[65]
Peach, Sweet cherry	*M. Fructicola*	Different maturity date	Unwounded	PDA	Drop 30 µL	1×10^5, 10^6	15 to 30 °C with 2.5 °C intervals, then at 20 °C/>95% RH, in plastic boxes	6 days	Disease severity (scaling 0 to 3) and percentage of fruit infection	[35]

Table 1. *Cont.*

Fruit Species	*Monilinia* spp.	Maturity Determination	Wounded or Unwounded (Intact)	Production of Inoculum	Mode of Inoculation	Inoculum Concentration (conidia/mL)	Incubation Condition	Assessment Time	Disease Assessment	Reference
Peach	*M. laxa*	Maturity at 0.6 I_{AD}	Unwounded	NA	Spray	1×10^5	Fruit left on the tree bagged in plastic or paper bags	7 days	Disease incidence% in the field	[14]
Peach	*M. laxa*	NA	Unwounded	NA	Spray	1×10^5	at 25 ± 2 °C/95–100% RH	7 days	Brown rot infection number, percent of rotted skin (lesion)	[50]
Peach, Nectarine	*M. laxa*	NA	Unwounded	NA	Sprayed to runoff	1×10^4, 10^6	23 °C/in trays lined with moist paper and plastic film. 16-h photoperiod	7 days	Incidence (%) of fruit rot	[4]
Peach, Nectarine	*M. laxa*	Optimum maturity	Unwounded, wounded	Peach fruit	Drop	25×10^3	23 °C/40–60% RH, in darkness	5 days	Measuring brown rot incidence (%), lesion diameter (mm) and colonization extent (mm)	[10]
Peach Apricot, plum	*M. laxa*	Commercial maturity	Unwounded, wounded	Fruit	Drop 20 µL	1×10^6	23 °C/high RH	10 days unwounded; 5 days wounded	Disease incidence, disease severity (lesion diameter)	[12]
Peach, Apricot	*M. laxa*	Commercial maturity	Unwounded, wounded	V8A	Dipping fruit for (1 min) inoculum	1×10^5	20 C and 95% RH	7 days	Brown rot incidence %	[58]
Peach, Plum	*M. laxa*	Mature fruit from the market	Wounded	PDA, canned peaches	Dipping for 30 sec in inoculum suspension or a drop	1×10, 10^2, 10^3, 10^4, 10^5 spore/cm^3	21 °C, wrapped in plastic bags	5 days, or 4 to 6 days	Disease incidence %	[93]

Table 1. *Cont.*

Fruit Species	*Monilinia* spp.	Maturity Determination	Wounded or Unwounded (Intact)	Production of Inoculum	Mode of Inoculation	Inoculum Concentration (conidia/mL)	Incubation Condition	Assessment Time	Disease Assessment	Reference
Apricot	*M. fructicola* *M. laxa*	Mature apricots	Unwounded, wounded	Tinned apricot halves	Drop 30 µL	1.5×10^4	15–22 °C	48, 66, 72, 96 and 120 h	Lesion area, spore counts, storage rot, cuticle thickness	[13]
Apricot	*M. laxa*	Mature visually	Unwounded	PDA	Drop (drip)	1×10^5	22 °C covered with polythene bags	7 days	Percentage infection and scaling to resistant: 0–10%; moderately susceptible: 11–30%; susceptible: 31−50%; highly susceptible: >50%	[53]
Sweet and sour cherry	*M. fructicola*	NA	Unwounded	PDA	Drop 30 µL	1×10^6, 10^5, 10^4, 10^3	20 °C/ 95%RH	6 days	Percentage fruit infection, lesion development	[66]
Sweet cherry	*M. fructicola*	Commercial maturity	Unwounded	NA	Spraying	1×10^4	13 °C 95–97% RH in the growth chamber	8, 11 days	Disease incidence	[54]
Sweet cherry	*M. laxa*, *M. fructigena*	5 to 6 weeks after blooming	Unwounded, wounded	PDA, Apple fruit	Spray	1×10^5	20 °C under light	7 days	Incidence of infection in field and polyethene tunnel	[56]
Prune	*M. fructicola*	Different stages	Wounded	Acidified PDA	Injecting ≈ 0.1 mL inoculum	5×10^3	Left on the tree	27 days or more	Disease incidence (%), and natural infection in the field	[100]

Abbreviations: V8A: V8 juice agar, PDA: potato dextrose agar, RH: relative humidity, NA: not available, I_{AD}: index of absorbance difference, ≈: approximately.

3.3. Inoculum Preparation

Inoculum suspension is prepared by flooding the culture plates or washing-off fruit with distilled water and wetting agent such as Tween 20 or 80 at 0.01% [11] or 0.05% [58] to scrape the conidia. Vigorous shaking or centrifugation of the suspension is needed to break conidial chains, followed by filtering through different means to reduce the mycelium parts in the suspension as much as possible. Strainers with pore sizes ranging from 25 to 40 µm, or layers of cheesecloth or lens tissue, among others, could be used. Finally, conidium concentration in the suspension is evaluated by counting aliquots by a hemocytometer or other counting chambers; the suspension is then adjusted to the desired concentration.

In literature, inoculum concentration ranged from 10^2 to 10^6 conidia mL^{-1} depending on fruit ripening stage or integrity (intact or wounded). In the case of ripe fruit, concentrations from 10^3 to 10^5 conidia mL^{-1} should not be exceeded to highlight resistance, as applying a high inoculum pressure would lead to generalized infections.

Immature fruit require higher concentrations (around 10^6 conidia mL^{-1}) to obtain significant infections; the suggested level is probably the maximum that could occur in field conditions when fruit are ripe [66]. Hong et al. [57] demonstrated enlargement of lesion diameter with increased inoculum concentration in wounded peaches; simultaneously, a concentration of 10^5 conidia mL^{-1} was required for unwounded fruit to obtain lesions around 10 mm diameter at 3-day post-inoculation. At lower concentrations (namely 10^2, 10^3, and 10^4 conidia mL^{-1}), fruit infections were delayed with significantly smaller lesion diameters. Overall, a concentration of 10^5 conidia mL^{-1} appears to be an effective inoculum concentration, particularly for inoculating intact fruit.

3.4. Field and Laboratory Protocols

In general, protocols can be divided into two categories: protocols applied in the field (or in-situ) and laboratory.

Field protocols are intended to quickly screen a high number of trees through the artificial inoculation of tree-attached fruit. Very few protocols are available for field evaluation. Luo et al. [100] inoculated tree-attached plum fruit by injecting 100 µL of *M. fructicola* conidia suspension at different growth stages and subsequently monitoring BR development. In a semi-field condition, Xu et al. [56] developed a protocol to evaluate the effect of fruit age and wetness duration on BR infection of tree-attached cherry fruit under polythene tunnel. A polythene bag was used to maintain adequate humidity; the inner side of the bag and the branch (including leaves and fruit) were wetted before inoculation by spraying distilled water. Then about 8 mL inoculum was sprayed onto the fruit on each branch until runoff, and then the polythene bag was placed over the branch and sealed with tape for different wetting periods before removing the bags.

More recently, in field conditions, Pacheco et al. [14] developed a protocol to screen large peach progenies in-situ to set up a more time- and cost-effective method to screen BR susceptibility in breeding programs.

Laboratory protocols provide a more accurate evaluation of the resistance displayed, although time-consuming as several steps are involved: fruit harvest, followed by preparation (as described in Section 3.1); arranging fruit in trays; inoculation, either on intact skin or after wounding in different ways. Inoculations by droplet or spray usually are practiced at different inoculum concentrations and incubation periods (see Section 3.7). Finally, observing fruit infection can be performed daily, and several indicators can be recorded (see Section 3.8). Both field and laboratory protocols have advantages and disadvantages (Table 2) and are contingent on the final objective and the quantity of material to be screened.

3.5. Wounded or Unwounded Fruit

Overall, injuring the fruit in the process of inoculation is a method to investigate the resistance of the flesh while infecting non-wounded fruit inspects the skin resistance. Since

fruit skin is the first barrier to fungal invasion [11], the resistance of the flesh is expected to be low; therefore, most of the studies focus on non-wounded fruit.

Several authors comparatively studied wounded and unwounded artificial inoculations (Table 1). Generally, stone fruit are successfully infected by both wounded and non-wounded methods, except for plums that appeared to be infected only by wounding [12,57,97].

Most of the studies show no correlation between skin and flesh resistance. As expected, unwounded fruit display less susceptibility, suggesting that most of the resistance lies in the skin [7,11,12,47,48,80,101]. Conversely, Mari et al. [58] observed a correlation between susceptibility of wounded and unwounded fruit in peach and apricot: they explained the results in light of a typical biochemical response of both skin and flesh. Finally, as evident in almost all literature, wounding deprives the fruit of its main barrier against pathogens [56,57], resulting in higher infection and severity levels compared to intact fruit.

Table 2. Advantages and disadvantages of field and laboratory-based protocols to evaluate fruit resistance level.

Evaluating Environments	Advantages	Disadvantages	References
Field	Relatively faster in manipulation. Plenty of accessions can be evaluated in a short time.	High variability, which may lead to low repeatability of the result. Environmental factors may impair the level of the recorded susceptibility.	[14,99]
Laboratory or controlled condition	Enables fruit preparation before inoculation, such as disinfection, wounding. Facilitates the post-inoculation evaluation of traits such as fruit weight, acidity, Brix. Provides repeatable environmental conditions. Fruit manipulations relatively easier. Inoculum load could be precisely placed on fruit sides (cheeks). Allows recording of many parameters.	Not exactly representing the natural (field) condition. It is more laborious.	[7,55,102]

3.6. Artificial Inoculum Application

Several methods have been used in artificial fruit inoculation, e.g., spraying, dropping, injecting, dipping. However, a comprehensive comparison among different methods is still lacking. Techniques are chosen based on their applicability and reliability in coherence with the whole protocol. For instance, spraying until inoculum runoff is mainly used in the field since other methods are difficult to apply on a tree-hanging fruit. Above all, this approach probably imitates the best way in which inoculum naturally arrives at multi-points on fruit in the field, via splashing [14,56,103].

In the laboratory, droplet fruit inoculation, dipping fruit in suspension, and fruit spraying are the main methods used for non-wounding fruit inoculation. On the other hand, some other methods are mainly used for applying inoculum to wounded fruit, such as placing or directly injecting an inoculum droplet and attaching an active mycelium plug to the wound (Table 1). The wounds can be made by inserting a disinfected needle or a sharp blade into the fruit peel. However, for both wounded and non-wounded, the position and amount of the inoculum are important and should be well maintained. The fruit cheeks are frequently chosen to deposit the drop inoculum, regardless of being wounded or non-wounded. At maturity, cheeks are considered the least susceptible fruit part to microcracking compared to suture, pedicel cavity, and stylar region, as reported for nectarine [71] and cherry fruit [104,105]. Some authors have explicitly considered

the position of depositing the inoculum droplet on the red (or sun-exposed) cheek [8,11]. The suspension amount per droplet may range from 10 to 30 µL regardless of inoculum concentration. Inoculation by paper disks soaked in a suspension of conidia and then laid on the fruit is a less common method [65]. Furthermore, non-ionic polysorbate such as Tween 20 or 80 at low concentrations from 0.005% to 0.05% is often added to the suspension [10,13,14,95], as surfactant (wetting agent) in conidial suspension. Its effects and functions have been widely studied on the inoculum preparation and viability of fungal biocontrol agents [101,106,107]. However, the influence of those surfactants on *Monilinia* has not been particularly addressed.

3.7. Incubation

Incubation is the time that *Monilinia* spp. requires to colonize inoculated fruit and display visible symptoms. However, this period may vary depending on the method of inoculation. For example, the time required to show the infection is shorter on wounded than non-wounded fruit; for peach, only two days are needed, while for plum, it takes four days [57]. Baró-Montel et al. [11] have measured lesion diameter at 3–7 days post-inoculation and observed a significant increase in lesion diameter in wounded fruit at 4 to 6 days post-inoculation. In contrast, for non-wounded fruit, the measurements were delayed up to seven days. Overall, most authors have considered seven days as an appropriate incubation period (Table 1).

Similarly, inoculated fruit can be incubated under the same conditions described in Section 3.2. Regardless of the stone fruit species, a diverse range of temperatures and humidity was used. However, predominantly inoculated fruit are incubated at ranges of 20–25 °C and 85–100% RH in a growth chamber or arranged in plastic boxes to secure the high relative humidity. On few occasions, fruit were incubated at lower humidity of 40–60% RH, which might not be optimal (Table 1). Furthermore, inoculated fruit are incubated at different photoperiods, such as continuous light or dark, and 12/12 h or 16/8 h light/dark photoperiods (Table 1). Since *Monilinia* spp. can successfully infect stone fruit at different light conditions, setting a photoperiod seems more reasonable. For example, 58 W white light in a 12/12 h light/dark cycle increased disease severity and sporulation more than continuous darkness in inoculated nectarines with *M. laxa*, while different photoperiods did not affect BR incidence [102].

3.8. Infection Assessment

Infection assessment is the final step of the phenotyping methodology when the state of infection is assessed and recorded. Two main variables are predominantly used: disease incidence and disease severity.

The disease incidence calculated as the number of infected fruits out of total inoculated. When the assessment is carried out in the field, this is the only available variable since no time follow-up is possible. Notably, disease incidence is the only variable recorded in cherry since measuring the progress of lesion diameter is difficult, given the small size of the fruit (Table 1).

The disease severity is estimated as the mean of lesion diameter or area, originated from inoculum point on the fruit surface. This is easier to record when only a single drop is deposited on the fruit, which is an indicator of the rapidity of the disease advancement (Table 1). Hong et al. [57] have not accounted for lesions that did not originate from the inoculation points since they were considered a natural infection. Furthermore, Biggs and Northover [35] have transformed the disease severity of unwounded peach and sweet cherry fruit inoculated with *M. fructicola* to a scale of 0 to 3, where 0 = no visible infection; 1 = necrosis not wider than the inoculum drop; 2 = necrosis wider than the width of the inoculum drop, but without sporodochia; and 3 = sporodochia present on the necrotic lesion.

Notably, BR development may be delayed in non-wounded compared to wounded fruit treatments [13]. In addition, a delay of lesion appearance and severity reduction is

reported when inoculum concentrations were lowered from 4×10^4 to 5×10^2 conidia mL^{-1} [103]. Consequently, for both cases, *Monilinia* spp. required more time to penetrate and develop on fruit.

In our lab, nine individuals of an interspecific peach progeny called BC2 were characterized through laboratory infection. We further took advantage of lesion diameter recording over time to investigate additional indicators in infection assessment, namely, the delay before infection and the speed of lesion appearance, which could be calculated from observed lesion profiles. The infection delay is considered as the time between drop deposit to the lesion formation (incubation period) by the naked eye, while the speed of lesion development was calculated as the maximum increase in lesion diameter in mm/hour. The infection diameter was calculated as the lesion diameter (mm) average recorded three days after infection.

The results exhibited distinctive variations between individuals and years of observations (2013 and 2014) (Figure 4). For most individuals, BR lesions were observed after 72 h (3 days). However, one accession particularly and to a lesser extent in addition to the infection was much more delayed or never occurred in many fruits, probably due to resistance factors at the skin level.

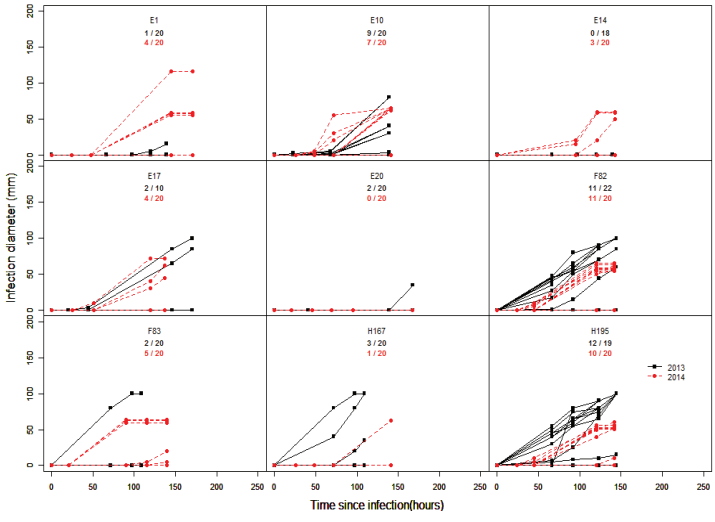

Figure 4. Development of lesion diameter (mm) in infected fruit. Intact fruit were inoculated with a droplet of 10 µL *M. laxa* at 10^5 conidia mL^{-1} concentration over two years. Each curve represents an infected fruit out of 20 inoculated (infected/total inoculated) fruits for nine genotypes (black curves: 2013; red curves: 2014). Inoculated fruit were incubated in clear plastic boxes with maximized relative humidity for seven days.

Regarding the lesion diameter and the speed of lesion development, they might be considered as linked to flesh resistance. These indicators scored on progenies or in germplasm collections can be regarded as quantitative traits and used in genetic analyses to explore trait-markers relationships.

4. Inconsistency of Infection Results

In plant biology, phenotypic instability is sometimes considered a form of plasticity in response to variations in environmental factors such as nutrients, water availability, and temperature [108]. Likewise, several hosts and related environmental factors may cause phenotyping inconsistency, in particular across years or methodologies. Pacheco et al. [8] noticed an inversion of the behavior of the two accessions 'Contender' and 'Elegant Lady'

for BR diameter between 2009 and 2010. In contrast, Martínez-García et al. [7] reported general consistency in ranking within a peach progeny over the three seasons tested. However, variation in resistance or susceptibility between two years was also reported.

Therefore, we further investigate the inconsistency of evaluation across years and methodologies in both orchard (field) and laboratory, including naturally occurring infections. The experiment was carried out on fruit of *P. davidiana* and *P. persica* cv Summergrand [109] for three consecutive years (2013, 2014, and 2015). A single-spore isolate of *M. laxa* (Ml3) at 10^5 conidia mL^{-1} inoculum concentration was used throughout the experiment. Mature fruit were inoculated by spraying in the field or applying a drop of 10 µL inoculum in the lab. Depending on the year, the genotypes were not stable in terms of susceptibility, regardless of the infection methodology. Low correlations were observed between years 2013 vs. 2014 and 2014 vs. 2015 in the field and between 2013 and 2014 in the laboratory. The best correlation was found in 2015 for the field vs. lab trials when inoculum spray was used. Additionally, field vs. natural infection was also significantly correlated in the year 2015 (Table 3).

Table 3. Correlation of brown rot disease incidence between years and different tests: lab and field inoculations were performed using drop and spray, respectively.

Inoculation Test	Correlation	*p*-Value
field 2013 vs. field 2014	0.2861	0.0063 *
field 2013 vs. field 2015	−0.0819	0.5004
field 2014 vs. field 2015	0.3148	0.003 *
lab 2013 vs. lab 2014	0.288	0.003 *
lab 2013 vs. lab 2015 [1]	−0.0292	0.8162
lab 2014 vs. lab 2015 [1]	0.2401	0.0522
field 2013 vs. lab 2013	0.2947	0.0056 *
field 2013 vs. lab 2014	−0.1151	0.3123
field 2013 vs. lab 2015 [1]	0.049	0.7223
field 2014 vs. lab 2014	0.1704	0.0884
field 2014 vs. lab 2015 [1]	0.2301	0.1509
field 2015 vs. lab 2015 [1]	0.4562	10^{-4} ***
field 2015 vs. natural infection 2015	0.3714	10^{-4} ***

[1] In 2015, lab inoculation was performed by spray. *** Significant differences at p value <0.0001, * Significant differences at p value <0.01.

In artificial infection in the field, environmental factors may be involved in this phenotypic diversity even though paper bags were used to maintain high humidity and favor infection [14]. The controlled inoculation mitigated the increased risk of infection, which is often observed as the season progresses. Interestingly, no correlation was found between the infection probability in the orchard and the different environmental factors (i.e., minimal and maximal temperature, moisture, wind velocity) (data not shown). As mentioned above, many factors related to fruit growing conditions, horticultural practices, and weather can be involved in such instability and are difficult to be accounted for. In the case of 'lab' tests, the year effect is complicated to explain, as usually this approach is supposed to control for several factors. Fruit developmental and growth conditions in the orchard undoubtedly play a significant role in this observed instability. For example, the status of the skin is essential, and perhaps the drop is sometimes deposited on microcracks not visible to the naked eye.

5. Conclusions

Phenotyping is a crucial step in breeding stone fruit for brown rot resistance. *Monilinia* spp. are necrotrophic fungi requiring several factors to infect stone fruit successfully. In the early stages of fruit development, success primarily depends on pathogen inocula and environmental conditions. Subsequently, other crop-related factors such as cultivar, fruit-related traits, and development stage, and field management practices play a significant role in BR development. As the main barrier to infection, fruit skin characteristics seem to be critical, also considering that microcracks and natural openings are the main entrance points of the pathogen. Despite many efforts initiated in breeding programs, more obviously for peaches and nectarines, no truly BR-resistant stone fruit cultivars are commercially available. While further attempts and contributions by stone fruit breeders are expected, the first step to success relies on the optimization of the phenotyping protocols. This literature review highlights the variability in applied procedures and non-consensus methodologies. All steps of the phenotyping protocol are crucial to ensure suitable infection performance, from fruit sampling to inoculum preparation and application. Fruit preparation before inoculation requires utmost attention: for example, when the natural pathogen pressure in the orchards is not too high, the best advice is not to disinfect fruit before inoculation. Furthermore, injuring the fruit seems to be a dead-end since an infection that has reached the flesh no longer stops. Moreover, the choice between spray in orchard and inoculum droplet in the lab lies in the objectives of the test and other variables to record and phenotyping capacity (workforce). Finally, it seemed that inoculum droplet and spray tests do not provide the same information regarding fruit susceptibility. Overall, even taking all possible precautions discussed, inconsistency could be expected, and multi-year assays are highly recommended to gather valuable results.

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Review

Advances and Challenges in RNA Interference Technology for Citrus Huanglongbing Vector Control

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Abstract: Citrus species, including sweet oranges, grapefruits, pomelos, and lemons, are the most widely cultivated trees and consumed fruits worldwide. In citrus orchard management, the control of Huanglongbing (HLB) disease and its insect vector *Diaphorina citri* (Asian citrus psyllid, ACP) represents a major global challenge. Consumers have been increasingly pushing the citrus production chain toward a more sustainable system, including stringent measures to prevent the use of chemical pesticides. In recent years, biotechnological advances have offered safe and environmentally friendly alternatives for crop production. Technologies such as RNA interference (RNAi)-mediated gene silencing have emerged as innovative tools for agricultural pest management. Here, we provide an overview of RNAi as a promising approach for ACP control and discuss the associated challenges. Despite the availability of specific silencing sequences aimed at a target gene of the insect pest, the uptake of double-stranded RNA is limited in hemipteran insects. In this context, improved delivery methods, stability maintenance, and RNAi response are considered the factors contributing to the increased effectiveness of exogenous RNAi against hemipteran pests. These approaches can serve as potential tools for efficient ACP control.

Keywords: gene silencing; Huanglongbing; sweet orange; crop protection; sustainability

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1. Introduction

Citrus juices act as the natural source of water, vitamins, minerals, sugars, acids, phytochemicals (e.g., polyphenols), and other organic compounds [1]. Owing to these characteristics, citrus species are the most popular fruit trees worldwide. In many countries, the citrus industry plays an important role in economic development, generating employment opportunities, increasing household income, and ultimately minimizing social inequality [2–5]. The conventional production methods of citrus fruits, such as oranges, mandarins, limes, and lemons, generate several products and by-products, which are primarily exported overseas [2].

Major obstacles in citrus tree development include weather conditions and disease epidemics. In particular, following its emergence, citrus Huanglongbing (HLB) disease has become the primary threat to citrus production in majority of the main global citrus-producing areas, including Brazil, the USA, and China [6–8].

HLB is caused by a Gram-negative bacterium of the genus *Candidatus* Liberibacter [9,10] and spread by the insect vector *Diaphorina citri* Kuwayama (Hemiptera: Leviidae), also known as Asian citrus psyllid (ACP) [11]. Within the plant, the bacterium is restricted to the phloem and cannot be cultured in artificial media. Moreover, there are no effective tools to control the spread of the insect vector and no commercial citrus varieties resistant to HLB have been developed to date, which further hamper disease control [6–8].

Nonetheless, integrated management involving the cultivation of trees in certified protected nurseries, monitoring of ACP populations and citrus tree shoots, application of biological and chemical measures to control insect population, and rapid removal of symptomatic trees from the field has been implemented [6,11,12]. Insecticides are a critical component of ACP management [11]; however, as insects can develop resistance to insecticides over time, new chemicals and protein-based molecules with different modes of action are essential to maintain disease control efficiency and provide sustainable solutions. In this light, biotechnological tools developed during the last two decades have proven useful in agriculture to overcome the various challenges of pest resistance, disease spread, weed control, and stress tolerance and thereby improve product yield and quality [13]. For instance, RNA interference (RNAi) is based on the silencing of target gene expression through the intervention of double-stranded RNA (dsRNA) [13]. This technology has been tested as a novel approach in different eukaryotic species and, thanks to its high specificity, may serve as a potent tool for crop protection by managing insect pests or controlling the spread of vector-borne diseases [14–16]. RNAi presents great potential for ACP management because most of the core machinery genes are present in insects [17]. Therefore, insect control can be achieved through the cultivation of genetically modified citrus plants or application of dsRNA-based pesticides, similar to the conventional ones [18]. However, the second approach can be considered easier for application in the field from the perspective of different regulatory processes across countries. RNA-based pesticides can protect trees while reducing the use of chemicals to control ACP [19], serving as an effective yet sustainable agricultural technology [20]. Despite its many benefits, however, some challenges related to the use of RNAi for insect control remain. For instance, lack of an efficient method for the delivery of dsRNA to the insect action site, off-target and non-target analytical effects, potential development of resistance, and cost of dsRNA synthesis are some of the shortcomings of RNAi [21–23]. In this mini review, we discuss the recent advances and major challenges in RNAi tools for ACP control.

2. RNAi Machinery

In RNAi, an RNA molecule inhibits mRNA translation, thus hindering protein synthesis [24]. Fire et al. [13] first described RNAi using dsRNA-mediated gene silencing in the nematode *Caenorhabditis elegans*. Briefly, once inside the cells, the dsRNA is cleaved by endonuclease III DICER into a small interfering RNA (siRNA) of 20–23 nucleotides. Then, the double-stranded siRNA is separated, and the guide strand binds to the Argonaute (AGO) protein, forming the catalytic component of the RNA-induced silencing complex (RISC) [24]. Next, the siRNA–AGO complex recruits other components of RISC and mediate mRNA degradation, mRNA translational repression, or chromatin modification, leading to post-transcriptional gene silencing (PTGS) [24].

The systemic effects of RNAi are essential for the practical application of RNAi-mediated silencing in pest and pathogen management. One of the pathways of systemic RNAi effects involves secondary dsRNA synthesis from the remaining mRNA molecule or the passenger strand of siRNA through the activity of RNA-dependent RNA polymerases (RdRPs) [25]. This secondary source of dsRNA has been reported in most eukaryotes, including plant pathogens and pests. However, no RdRP orthologs have been detected in hemipterans, suggesting that secondary dsRNA synthesis does not occur in these insects and that they present another as yet unreported system that amplifies dsRNA synthesis [16]. Although the precise mechanism underlying the amplification dsRNA synthesis remains unknown, several studies have reported the success of RNAi-mediated silencing in hemipterans, including ACP. Given that these insects harbor the core components of the RNAi machinery, namely DICER and AGO family proteins, RNAi-mediated gene silencing may indeed be used to manage ACP [26].

3. Challenges in RNAi-Mediated Silencing for Pest Control

RNAi-mediated silencing is a promising tool for pest control. However, for efficient dsRNA action in insects, the molecule must be taken up by the intestinal lumen and come in contact with the interior of the cell, where it can trigger PTGS [16]. However, certain challenges are involved in the uptake of dsRNA and activation of RNAi machinery, such as the dsRNA delivery method, dsRNA concentration, dsRNA nucleotide sequence and length, dsRNA persistence inside the insect body, and developmental stage of the insect.

3.1. dsRNA Uptake and Spread into the Insect Body

dsRNA can be taken up through two mechanisms: cell-autonomous and non-cell-autonomous. In the cell-autonomous mechanism, dsRNA-triggered silencing is restricted to the cells in which the dsRNA is introduced or expressed. This mechanism is the most common RNAi pathway and involves the cleavage of dsRNA into siRNA through RISC to induce PTGS [24]. In the non-cell-autonomous mechanism, dsRNA-triggered silencing occurs in cells or tissues different from those where the dsRNA is introduced or synthesized. The non-cell-autonomous mechanism involves environmental and systemic RNAi. Environmental RNAi refers to all mechanisms through which dsRNA is absorbed by the cell directly from the surrounding environment (eRNA) [27,28], while systemic RNAi involves the transport of dsRNA molecules from one tissue or cell to another, occurring exclusively in multicellular organisms [29,30].

The internalization of dsRNA/siRNA by cells is essential for RNAi-mediated silencing in insects. If dsRNA is taken up via ingestion, the molecules must pass through the peritrophic matrix before being internalized by the midgut epithelia and bind to lipophorins in the hemolymph [31]. The systemic effect of RNAi-mediated silencing is not well known in insects, although it has been well documented in the nematode *C. elegans* [30].

The injection of dsRNA into *C. elegans* gut lumen can allow for the determination of whether the dsRNA/siRNA is spread throughout the cell via the action of systemic RNA interference deficient-1 (SID-1). SID-1 is a transmembrane protein that acts as a channel for dsRNA uptake via diffusion, and this protein is present in all cells sensitive to RNAi-mediated silencing [29,30,32]. Although the systemic effect of RNAi is SID-1-dependent, dsRNA uptake is SID-2-dependent. SID-2 is a transmembrane-like-protein localized in the gut lumen cells of animals. This protein is important for the incorporation of RNA from the environment into the organism but plays no role in the transport of the endogenous molecules [28]. Thus, SID-1 and SID-2 proteins or their orthologs are essential for RNAi systemic effects and dsRNA uptake from the environment, respectively, enabling gene silencing [28,29,32]. SID-1 protein homologs have been reported in several insect species, such as the red flour beetle (*Tribolium castaneum*), silkworm (*Bombyx mori*), European honey bee (*Apis mellifera*), aphids (*Myzus persicae*) [33], and ACP [26].

Hemipterans, coleopterans, dipterans, and orthopterans lack SID homologs, and dsRNA uptake in these insects likely occurs through receptor-mediated clathrin-dependent endocytosis. In this mechanism, dsRNA is internalized via the action of a scavenger receptor [31]. In *T. castaneum*, despite the presence of SID protein homologs, endocytosis is the major pathway of dsRNA uptake. In this mechanism, dsRNA first binds to unknown receptor proteins in the plasma membrane. The resulting complex, called AP-2, interacts with clathrin to form a clathrin-coated vesicle, which fuses to an endosome. Before the fusion of the late endosome to a lysosome, dsRNA molecules are released into the cytosol, where they trigger the RNAi core machinery [31,34,35]. Thus, the endocytotic mechanism of dsRNA uptake may promote RNAi-mediated gene silencing in organisms. In ACP, the core components of clathrin-mediated endocytosis, namely the clathrin heavy chain and AP50, an AP-2-like protein, have been identified [17].

3.2. dsRNA Delivery

dsRNA can be delivered into ACP through several means, and the primary delivery methods can be divided into transformant and non-transformant methods (Figure 1) [36].

The transformant method involves the generation of a transgenic plant (genetically modified organism, GMO) harboring a construct to produce dsRNA targeting the insect mRNA. Meanwhile, the non-transformant delivery methods are GMO-free and include micro-injection of dsRNA directly into the hemolymph [37], topical application on insect body [38,39], soaking insects into a dsRNA solution [40,41], feeding insects with artificial diets containing dsRNA molecules [42–44], and feeding insect with plants treated with a dsRNA solution through drenching or trunk injection [45]. Despite the introduction of several dsRNA molecules, however, only those delivery methods that result in insect topical absorption and/or acquisition via feeding are applicable for ACP management in citrus orchards (Table 1). Thus, the most suitable dsRNA delivery methods for ACP control involve foliar spray, root absorption via irrigation water, and ingestion of GMO plants expressing dsRNA for insect target gene silencing [46].

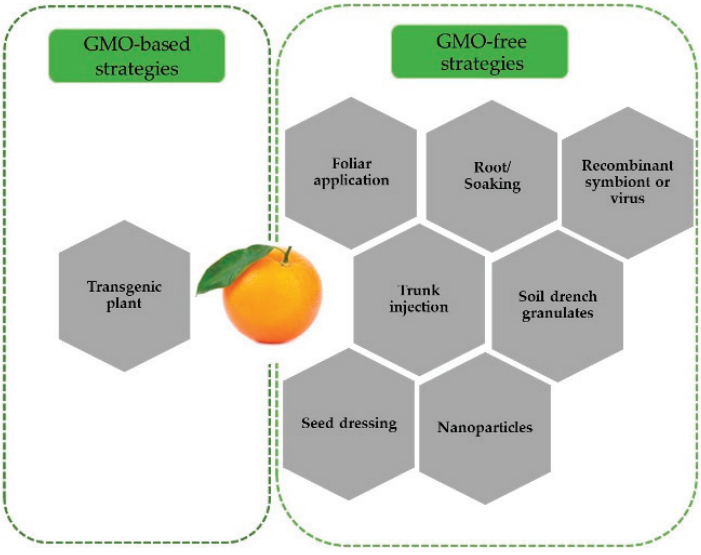

Figure 1. Novel approaches to RNA interference (RNAi)-mediated insect control—delivery strategies. GMO-based strategies: use of genetically modified plants is the best-known approach to RNAi-based control of insect pests or vectors. GMO-free strategies: specific genes of target in an insect are silenced using novel approaches.

However, the dsRNA delivery methods involving topical absorption and ingestion present some additional challenges, because in both approaches, the dsRNA molecules must overcome additional barrier mechanisms expressed by the insects to prevent the internalization of the molecules in cells. For instance, in topical application, the dsRNA must pass through the covering ACP tissues, penetrating the cuticle layers, until reaching the epidermis cells [47]. In dsRNA delivery methods relying on absorption during the feeding period, the dsRNA molecules must remain stable under the conditions of the gut long enough to be absorbed into the epithelium cells and must be transported throughout the insect body, taken up in distant cells, and then processed or used to suppress complementary mRNAs [31,48]. In some hemipteran pests, oral delivery is confounded by high ribonuclease activity in the saliva, which degrades dsRNA [49].

Additionally, the delivery methods that involve dsRNA acquisition via ingestion are associated with the additional challenges of the availability of dsRNA or siRNA in GMOs at amounts sufficient to trigger RNAi-mediated silencing response [35]. In GMOs, majority of the dsRNA molecules delivered are siRNAs, which may affect the stability of the molecules inside the insect body, as smaller molecules are more easily degraded by nucleases in the

midgut [27,50–52]. In contrast, non-transformant methods only involve the acquisition of longer dsRNA by insects [27]. Nonetheless, hemipterans, such as ACP, are sap-sucking insects, and the dsRNA must be available at sufficient amounts in the tissues on which the insects mainly feed, such as the phloem. Thus, for ACP control, dsRNA delivery methods should provide dsRNA molecules into the phloem vessels.

Table 1. Asian citrus psyllid (ACP, *Diaphorina citri*) control via RNAi technology.

Target Gene	Gene Target Function	dsRNA Delivery	Developmental Stage	Result	Reference
Abnormal wings disc (awd)	Essential for adult wing formation	Topical application	Fifth instar nymphs	Increased nymph mortality and abnormal wing development in adults	[53]
Cytochrome P450 gene family 4 (CYP4)	Detoxifying enzyme related to insecticide resistance	Topical application	Adults	Increased insecticide susceptibility	[54]
Arginine kinase	Energy mobilization	In plant system	Adults	Increased adult mortality	[26]
Superoxide dismutase (SOD)	Antioxidant defense	In plant system	Adults	Increases adult mortality	
Pterin-4-alpha-carbinolamine dehydratase (PCDB1)	Cell metabolism	In plant system	Adults	None	
Tomosyn *Vitellogenin*	Nervous system Reproduction	In plant system In plant system	Adults Adults	None None	
Cathepsin D	Metamorphic events	Ingestion through artificial diet and in plant system	Adults and fifth instar nymphs	Increased insect mortality	[55]
Chitin synthase	Chitin synthesis	Ingestion through artificial diet and in plant system	Adults and fifth instar nymphs	Increased insect mortality	
Inhibitor of apoptosis	Regulation of the apoptotic machinery	Ingestion through artificial diet and in plant system	Adults and fifth instar nymphs	Increased insect mortality	
Esterase FE4-like (EstFE4); acetylcholinesterase (ACh)	Detoxifying enzyme related to insecticide resistance	Topical feeding	Fourth and fifth instar nymphs	Increased mortality of nymphs and, consequently, of emerged adults	[56]
Muscle protein 20 (*DcMP20*)	Encodes a cytoskeletal protein	Soaking	Third and fourth instar nymphs	Increased nymph mortality	[41]
Abnormal wings disc (awd)	Essential for adult wing formation	Topical feeding	Adults and fifth instar nymphs	Increased nymph mortality and deformed adults	[39]
Boule	Fertility	Feeding in artificial diet	Adults	Increased adult mortality, but no effect on number of eggs	[42]
Sucrose hydrolase (DcSuh)	Enhanced absorption of sucrose from the midgut	Topical application	Fifth instar nymphs	Increased nymph mortality and shortened insect lifecycle duration	[38]

Table 1. *Cont.*

Target Gene	Gene Target Function	dsRNA Delivery	Developmental Stage	Result	Reference
Laccase-1-S	Detoxification of secondary plant compounds in insects	In plant system	Adults	None	[57]
DcSnf7	Transport of proteins for degradation via the endosomal autophagic pathway	In plant system	Adults	None	
Calreticulin	Calcium ion chelation, which helps maintain phloem circulation	In plant system	Adults	None	
Trehalose-6-phosphate synthase	Energy synthesis; metamorphosis	Feeding in artificial diet	Fifth instar nymphs	Increased nymph mortality and deformed adults	[43]
Odorant-binding 7	Host plant volatile compound recognition	Ingestion through artificial diet	Adults	Suppressed electro-physiological responses of antennae and disrupted insect behavioral responses	[44]

Currently, in RNAi experiments with ACP, the so-called "in plant systems" (iPS) are commonly used to deliver dsRNA into psyllids via feeding [15]. This delivery method involves the use of a detached leaf that absorbs a solution containing the dsRNA for gene silencing in insects via the petiole. Although this method with a Cy3-labeled dsRNA has been successfully used by Taning et al. and Galdeano et al. [26,55], Angelotti-Mendonça et al. [57] demonstrated that most of the dsRNA absorbed by the detached leaves remained in the xylem. Because ACP mainly feeds on sap in the phloem and only derives water from the xylem, the insects likely ingested only a small amount of dsRNA from the xylem. Thus, iPS is not suitable to silence the target candidate genes in ACP using RNAi. Moreover, this method does not properly simulate dsRNA application methods, such as drenching or foliar spray. For instance, in citrus nursery trees, dsRNA delivery through drenching resulted in dsRNA absorption by roots and its spread to other plant tissues, including phloem, as evidenced by the detected of labeled dsRNA in the xylem and phloem. Additionally, dsRNA molecules were detected in the vessel tissues of new shoots and leaves of the treated citrus nursery trees 3 days after the treatment, indicating that dsRNA delivery through irrigation water may be suitable for ACP control in citrus orchards, because a large amount of dsRNA was detected in the phloem [58].

The most common method to deliver pesticides is through foliar spray; however, the application of this method to deliver dsRNA for the control of sap-sucking insects involves additional challenges. In this method, the insect may take up dsRNA molecules through contact, similar to soaking or topical application, or via feeding on plants. Following the absorption and spread of dsRNA molecules to the phloem vessel tissues, they must remain stable for the required period and be available at amounts sufficient to trigger silencing until they are consumed by the insects. Additionally, in foliar spray, the translocation and uptake of dsRNA must overcome several barriers, such as passing through the leaf cuticle, degradation by nucleases until absorption by the vegetal cells, and spreading into phloem vessel tissue [59].

3.3. dsRNA Stability and Efficiency

The stability and efficiency of RNAi for gene silencing in insects may drastically vary depending on the concentration or dose and the length or size of the dsRNA, application method, delivery technique, plant organospecific processes, adverse environmental conditions, insect life stage, and choice of the target gene to be silenced [19,60].

For most insects, the results of dsRNA injection delivery differed from those of the other delivery methods. dsRNA injection enables the delivery of a precise amount of dsRNA directly into the target tissues or the hemolymph, consequently achieving a high level of gene silencing [17,56,61]. The first contact before cellular uptake is either the hemolymph or the gut content [31], and these body fluids may act as the main causes of dsRNA instability due to the enzymatic activity of double-stranded ribonucleases (dsRNases) and the physiological pH driving enzymatic activity in these fluids [31,35]. Additionally, the lack of functional RNAi machinery and availability of limited knowledge on dsRNA uptake, transport, and systemic propagation mechanisms contribute to this instability [35]. The ACP genome harbors three dsRNase homologs, and dsRNA degradation due to high pH and/or dsRNase action in the gut and salivary secretions has already been confirmed [15]. Recently, dsRNA degradation was confirmed when a high dsRNA dose was required to suppress the expression of the *DcBol* fertile gene [17,42].

Some strategies have already been developed to address issues related to dsRNA instability. As such, nanotechnological applications can significantly enhance the efficiency of agricultural inputs, such as plant growth promoters, nano-pesticides, nano-fertilizers, nano-herbicides, agrochemicals, and RNAi-based pesticides [62]. The nanoparticle-mediated dsRNA delivery system has been used to improve the persistence, penetration, and transport of RNAi molecules into plants or insects [35,63].

Nanoparticles are matter particles ranging in diameter between 1 and 100 nm. Advantages of using chitosan, liposomes, and cationic dendrimers as nanoparticles for protecting and delivering dsRNA/siRNA to improve RNAi efficiency and thus promote the development and practice of RNAi-based pest management strategies have already been documented [61]. In addition, in recent years, carbon-based nanomaterials, such as carbon nanotubes, and the structure–property–cytotoxicity interplay of other nanoparticles, such as quantum dots and graphene oxide, have been investigated [64,65].

Recently, the combination of RNAi and nanotechnology was demonstrated efficient in increasing the stability and efficacy of dsRNA in RNAi-mediated bed bugs control [66]. For bed bugs, the use of a long dsRNA elicited a silencing response; however, a high dosage was required to obtain a similar response in other insects, such as coleopterans [67]. This difference may be related to taxon-specific RNAi response. In coleopterans, an orally delivered long dsRNA ($\geq$200–500 nucleotides) is processed into the cell to yield siRNAs, which ultimately trigger mRNA degradation [66]. The dsRNA fragment size may be reduced to avoid off-target silencing [68,69]. In addition, protection by nanoparticles increased the efficiency of RNAi action in *Euchistos heros* [66]. To trigger RNAi in *E. heros*, short hairpin RNA (shRNA) was orally delivered using cerium oxide nanoparticles (4 nm) coated with diethylamioethyl dextran (as the carrier. The particle properties and shRNA loading rates (Ce:shRNA mass ratio) resulted in successful transcript reduction or RNAi. Significant mortality attributed to RNAi was observed at the shRNA concentration of 250 ng·μL^{-1} in the feeding solution. The degradation of the targeted troponin transcript via nanoparticle-delivered shRNA was equivalent to that observed with a long dsRNA, although naked shRNA transcript reduction was not statistically significant [66].

Nonetheless, these nanoparticle-based RNA pesticides must be simple and the required raw materials should be affordable for low-cost production [61,70]. In addition, application dsRNA-based pesticides through either irrigation or foliar spray must be explored [68] to promote their oral uptake by insects via feeding [15].

Although nano-formulations are in high demand in the pesticide industry, there is little information on the environmental risks associated with nanoparticle-based RNA

pesticides, and further research is on the environmental impact assessment of nanotools is warranted [61,71].

3.4. Off-Targets

With accumulating knowledge on the RNAi machinery, novel approaches using this technique have been elucidated and developed every year [18,19,31,60,72,73]. siRNAs of approximately 21–25 nucleotides are the key intermediaries in triggering sequence-specific RNA degradation during PTGS in invertebrates [72]. These sequence specificities minimize any detrimental effects of RNAi on non-target organisms (NTOs) [74].

In recent years, there has been significant development in bioinformatic software to assess the potential hazards of RNAi. Software have been developed to optimize the RNAi design to ensure specific target gene knockdown [75]. To date, however, there are methods to quantify the reliability of these bioinformatic software in assessing such off-target effects. Given the variability in environmental exposure, barriers to systemic exposure, and differences in RNAi machinery among organisms, the bioinformatic software as the sole tool is not recommended [74,76,77]. Specialists from the academic, industrial, and government sectors have discussed several aspects of RNAi, with particular focus on furthering our knowledge of the technology for risk assessment [18,78]. The conference held by the Organization for Economic Cooperation and Development covered multiple perspectives related to RNAi-based pesticides. The potential exposure of NTOs and responsiveness to environmental RNAi have been discussed as the main parameters to be considered in the risk assessment of exogenous dsRNA application, and appropriate legislation involving risk assessment procedures in science-based topical RNAi-based applications must be enacted (Figure 2) [76].

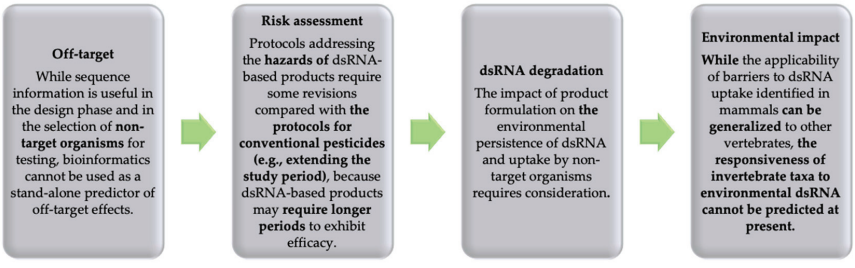

Figure 2. Key aspects and considerations related to the use of RNAi-based pesticides discussed at the conference held by the Organization for Economic Cooperation and Development (OECD) (adapted from Mendelsohn et al. [76]).

A recent study evaluated the activity of three specific dsRNA molecules targeting genes in different insects, representing the classic biocontrol agents of the emerald ash borer (EAB, *Agrilus planipennis*) in some countries. The study provided substantial data to demonstrate the feasibility of using novel alternatives for EAB management that are ecofriendly and safe for NTOs [74].

4. ACP Control Using RNAi Technology

Since the first report of the use of RNAi-mediated gene silencing for pest control [79,80], the application of this technology has been extensively studied for the control of insects, including ACP. Indeed, the success of this technology depends on several factors that affect the dsRNA delivery method, and target gene selection is crucial. Several studies on the RNAi-mediated control of ACP are summarized in Table 1. Additional factors including the characteristics of the dsRNA molecule, such length and concentration, must also be appropriately adjusted to ensure pesticide effectiveness.

Initial studies on the RNAi-mediated control of ACP focused on the delivery of dsRNA via topical application. In the first study using dsRNA, the *abnormal wing disc* (*awd*) gene,

which is essential for adult wing formation, was successfully silenced through topical dsRNA application in fifth instar nymphs, leading to wing deformation and flight arrest in adults [53]. Successful silencing of several target genes at different insect developmental stages using various dsRNA delivery methods has been tested (Figure 1).

RNAi-mediated ACP control has also been tested using feeding assays. Through the ingestion of an artificial diet, several genes including *cathepsin D*, *chitin synthase*, *inhibitor of apoptosis* [55], *boule* [42], *trehalose-6-phosphate synthase* [43], and *odorant-binding 7* [44], in ACP were silences, leading to a lethal phenotype and/or an abnormal behavioral response. Furthermore, knockdown of the *arginine kinase* and *superoxide dismutase* genes through feeding and *iPS* reduced the ACP adult survival rate [26]. In some other studies, despite successful gene silencing using *iPS*, however, dsRNA delivery through this method did not affect the insect survival rate or the expression of *pterin-4-α-carbinolamine dehydratase*, *tomosyn*, *vitellogenin* [26], *laccase-1S*, *calreticulin*, and *Snf7* [57]. These contrasting results suggest that the RNAi-mediated ACP control also depends on target selection. Additionally, successful *Snf7* silencing in Western corn rootworm (*Diabrotica virgivera*) [81] and lack of effect in ACP indicate that silencing of a target gene in one organism does not necessarily work in others.

The dsRNA characteristics and delivery methods as well as the developmental stage of the insect treated with dsRNA can directly affect RNAi-mediated gene silencing. In general, hemipterans present stronger ribonuclease action at the adult stage, leading to higher dsRNA degradation and greater insect resistance when the dsRNA is delivered through oral ingestion [35,82–84]. In ACP, the early developmental stages (e.g., nymphs) exhibit greater sensitivity to RNAi than the later developmental stages (e.g., adults) [41]. Indeed, topical dsRNA application resulted in successful target silencing of the *awd* gene [53]. In addition, the mortality of third and fourth instar nymphs increased after soaking them in a dsRNA solution targeting *muscle protein 20* (*DcMP20*) [41]. In addition, at the juvenile stages, topical dsRNA application resulted in the silencing of *sucrose hydrolase* (*DcSuh*), which reduced survival rate of the fifth instar nymphs and shortened the insect lifecycle duration [38]. Therefore, the application of RNAi-mediated control at the early developmental stages of ACP is essential for HLB management in citrus orchards. The fourth and fifth instar nymphs of ACP can spread the causal agent of HLB and increase the disease transmission, by spreading the pathogen as both nymphs and adults [85].

In addition to the development stage, the potential targets must be differentiated for a specific insect stage. Moreover, knowledge of the biological interactions with the host is essential to increase the efficiency of silencing of the candidate genes and, particularly, to ensure the reproducibility of the technology in the field. For instance, a recent study explored the molecular mechanism of the reproductive strategy of ACP. The fecundity and ovarian morphology of females confined to young shoots and mature citrus tissues were evaluated. In ACPs feeding on young shoots, psyllid female ovarian development was activated, inducing oviposition. However, silencing of the positive regulatory gene *DcRheb* in the target of rapamycin (TOR) pathway lowered ecdysone and juvenile hormone levels, suppressed vitellogenin synthesis, and reduced female reproductive capacity [86]. Genes in the TOR pathway, among others, have been proposed as the potent candidates for silencing to reduce the reproductive capacity of insects; however, if product of choice is an RNAi-based pesticide, it must be applied to shoots for successfully silencing the target transcripts.

5. Primary Considerations and Future Prospects of RNAi Strategies for ACP Control in Citrus Orchards

RNAi can serve as a potentially biosafe strategy to manage ACP. It can help control this insect pest through two modes of application: non-transformative (bioproduct) and transformative (transgenic plants) delivery. The non-transformative delivery presents some advantages for use in pest management because of the rapid product development, low cost, low resistance risk, and high viability for all crops [87], although there are no stringent regulations in different countries [88].

In this context, innovation systems in food and agriculture actively seek to go beyond the financial targets [89]. Building a more sustainable citrus industry for the future is essential to conserve the environment as well as respond to the demands of the food sector, which are increasingly focused on the environmental and social aspects. Therefore, exploiting cutting-edge technologies such as RNAi is essential to reduce the use of pesticides in orchards, thus generating minimal environmental impacts [35] and avoiding adverse effects on NTOs, such as pollinators. Finally, investment in scientific research is increasingly playing a relevant and significant role in promoting the citrus industry and ensuring that citrus growers continue to supply good quality fruits to the consumers and processors, overcoming the most relevant challenges in cultivation [2].

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