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Effect of Organic Additives on the Micropropagation of *Asparagus officinalis*

Preekamol Klanrit [1,2], Khanittha Lila [3], Paramaporn Netsawang [3], Phakamas Siangsanor [3], Pornthap Thanonkeo [1,2] and Sudarat Thanonkeo [4,*]

[1] Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand; kpreek@kku.ac.th (P.K.); portha@kku.ac.th (P.T.)
[2] Fermentation Research Center for Value Added Agricultural Products (FerVAAPs), Khon Kaen University, Khon Kaen 40002, Thailand
[3] Agricultural Promotion and Development Centre Suphan Buri, Suphan Buri 72160, Thailand; tha_402@hotmail.com (K.L.); paramaporn.n@rice.mail.go.th (P.N.); faii02fai@gmail.com (P.S.)
[4] Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham 44150, Thailand
* Correspondence: sudarat.t@msu.ac.th; Tel.: +66-862268160

Abstract: In vitro propagation is one of the most promising techniques for the large-scale clonal propagation of *Asparagus officinalis*. The aim of this study was to investigate the effect of organic additives, including coconut water (CW), banana homogenate (BH), and potato homogenate (PH), on *A. officinalis* shoot and root proliferation. The results revealed that CW, specifically at 20% (*v*/*v*), was the most effective organic additive for promoting shoot and root formation in the in vitro plantlets. Furthermore, the longest shoot and root lengths were also observed in the MS medium supplemented with 20% (*v*/*v*) CW. In the supporting medium of 1:1 peat moss and vermiculite, the in vitro plantlets exhibited a high survival rate with a morphology comparable to that of the mother plant. The results of this study demonstrate that CW can be applied as a supplemental material for large-scale *A. officinalis* micropropagation.

Keywords: *Asparagus officinalis*; organic additive; plant tissue culture; plant growth regulator

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1. Introduction

Asparagus is one of the most important genera of the family Asparagaceae, comprising approximately 200 species of herbaceous perennial and tender woody shrubs that are native to Asia, Europe, and Africa [1,2]. Among the different species of asparagus, garden asparagus (*Asparagus officinalis* L.) is the most important in terms of economic, human nutritional, and medicinal value [3,4]. The aerial parts, roots, and seeds of *A. officinalis* contain several bioactive compounds, such as saponins, vitamins, flavonoids, steroids, glutathione, and spirostanol glycoside, which possess a variety of biological properties, including antioxidant, anti-inflammatory, antihepatotoxic, antimicrobial, antidiarrheal, antiulcerogenic, and anticancer properties [5–11].

A. officinalis is cultivated worldwide, and global production is estimated to reach 10.7 million metric tons by 2026. China is the world's largest producer of asparagus at approximately 7.3 million metric tons in 2021 [12]. Conventional propagation of *A. officinalis* is performed using seeds or sometimes vegetatively via separation of the plant rhizome. However, despite the low viability of plant seeds, the slow growth rate of plantlets obtained from seed propagation, and the risk of spreading diseases, such as anthracnose, soft rot, and *Fusarium* rot, vegetative propagation [4,13] is not often used in commercial production. In vitro propagation or micropropagation techniques, such as direct or indirect organogenesis and somatic embryogenesis, can be used to overcome these obstacles. These propagation techniques provide large uniform plantlets with genetic stability; they are

considered cost-effective methods for commercial propagation and can eliminate systemic diseases caused by plant pathogens.

In vitro propagation of *A. officinalis* has previously been reported, and most studies have focused on the effects of plant growth regulators (PGRs) on plant regeneration and development. For instance, Esmaeili et al. [3] investigated the effect of PGRs on the in vitro regeneration of *A. officinalis*. The authors reported that the highest number of shoots (4.25 shoots/explant) and roots (2.0 roots/explant) were obtained with Murashige and Skoog's (MS) medium supplemented with 1.5 mg/L 6-benzylaminopurine (BAP) and 0.05 mg/L 1-naphthaleneacetic acid (NAA) and with 0.4 mg/L indole-3-butyric acid (IBA), respectively. Rasad et al. [14] reported the in vitro propagation of *A. officinalis* using the stem and root explants of sterile plantlets. The authors reported that MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA resulted in the highest number of shoots (6.233 shoots/explant and 4.967 shoots/explant for stem and root explants, respectively). The highest number of roots was observed in MS medium supplemented with 0.5 mg/L BAP and 1.5 mg/L NAA for microshoots regenerated from stem explants and in MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA for microshoots regenerated from root explants. Another study by Maung et al. [15] reported that MS medium supplemented with 2.0 mg/L BAP produced the highest values for the number of shoots (9 shoots/explant), shoot length (2 cm), and number of nodes (2 nodes/shoot), while the highest rooting rate (40%) was observed in medium supplemented with 2.5 mg/L IBA. Most recently, Minh et al. [16] demonstrated that MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA resulted in the highest shoot formation rate (87.2%), while MS medium supplemented with 0.5 mg/L NAA yielded the highest rooting rate (74.59%) of in vitro *A. officinalis* plantlets.

The cultivation media for in vitro plant propagation comprise macro- and micronutrients, amino acids, vitamins, carbon sources, PGRs, and gelling agents, which are generally expensive [17]. Therefore, modifying the cultivation media composition by adding low-cost organic substances to replace expensive materials is considered an efficient approach for large-scale clonal plant propagation [18]. A variety of organic additives, such as coconut water (CW), banana homogenate (BH), potato homogenate (PH), yeast extract (YE), tomato juice (TJ), pineapple juice (PJ), and pineapple pulp (PP), have been widely used as supplements in plant tissue culture medium [18]. These organic additives provide not only natural sources of carbon, such as sucrose and fructose, but also natural vitamins, amino acids, lipids, minerals, and PGRs, specifically plant hormones, which are essential for plant growth and development [19,20]. However, based on a literature review, the effect of organic additives on the in vitro propagation of *A. officinalis* has never been documented; in most studies, organic additives were added to culture media for the in vitro propagation of orchids [20–22]. Therefore, to overcome the limitations of the conventional propagation of *A. officinalis*, which is generally based on plant seeds and rhizomes, and to minimize the in vitro propagation cost of plant tissue culture medium, this research aimed to employ an in vitro culture technique for the micropropagation of *A. officinalis*. Furthermore, the effect of low-cost and readily available organic additives, including CW, BH, and PH, on in vitro shoot and root proliferation in *A. officinalis* was also evaluated. The present study could provide useful information for the sustainable large-scale production of *A. officinalis* plantlets.

2. Materials and Methods

2.1. Chemical and Explant Preparation

Chemicals, including the plant tissue culture medium (Murashige & Skoog (MS) basal medium), phyto agar, and plant hormones (NAA and Kinetin (N6-furfuryladenine) were acquired from PhytoTech Labs, Lenexa, KS, USA, and Sigma Aldrich Corporation, Burlington, MA, USA, respectively. CW, banana (cv. Hong Thong), and potato were purchased from the local market in Khon Kaen, while peat moss and vermiculite were procured from Bee Garden and Farm Co., Ltd., Khon Kaen Province, Thailand.

Two-year-old plants of *A. officinalis* cultivar Brock's Improved were used in this study. Plants were collected and washed thoroughly with running tap water, and then the stems of the plants were cut to approximately 5 cm long. For surface sterilization, the explants were washed with 70% (*v*/*v*) ethanol for 1 min and then washed three times with sterilized distilled water to remove the excess ethanol. The resulting explants were soaked in 15% (*v*/*v*) sodium hypochlorite for 15 min, followed by 10% (*v*/*v*) sodium hypochlorite for 10 min. After washing with sterilized distilled water, the axillary buds were excised and placed on MS basal medium supplemented with 1.0 mg/L kinetin and 0.05 mg/L NAA and cultured in a standard culture room at 25 ± 2 °C with a light intensity of 3000 lux and a 16-h photoperiod. The explants were subcultured on the same fresh solid MS basal medium before being cultivated for 28 days under the specified conditions.

2.2. Preparation of Organic Additives and Culture Medium

CW was taken directly from a young tender coconut. After filtration through four layers of cheesecloth to remove impurities, such as coconut bark or palm kernel, it was stored at −20 °C before use. The ripe banana was used in this study. The peeled fruit (100 g) was cut into small pieces and ground using a fruit blender (HR2115, Philips, Bangkok, Thailand). The resulting BH was collected and kept at −20 °C for future use. To prepare PH, 100 g of peeled potato was cut into small pieces and ground using a fruit blender. The resulting homogenate was collected and stored at −20 °C before use.

The culture medium was prepared by dissolving 4.43 g MS basal medium and 30 g of sucrose in distilled water. CW, BH, or PH at various concentrations was added, and the pH of the medium was adjusted to 5.8 using 0.1 N NaOH/HCl. Seven grams of phyto agar powder was added, and the volume of the culture medium was adjusted to 1 L using distilled water. After boiling using a microwave, 30 mL of the medium was aliquoted into a culture vessel and later sterilized by autoclaving at 121 °C, 15 kPa for 15 min.

2.3. Effect of Organic Additives on Shoot Proliferation

The sterile explants of *A. officinalis* were placed on MS basal agar medium containing 0.05 mg/L NAA and supplemented with different concentrations of CW (5, 10, 15, and 20%, *v*/*v*), BH (5, 10, 15, and 20%, *w*/*v*), and PH (5, 10, 15, and 20%, *w*/*v*). The MS basal medium was used as a control without PGRs and organic additives. After 4 weeks of cultivation, the number of shoots and the average shoot length were measured. One explant was placed in each culture vessel. All experiments were carried out twice, with ten replicates for each treatment.

2.4. Effect of Coconut Water on Root Proliferation

The sterile explants (stems) of *A. officinalis* were placed on MS basal agar medium supplemented with different concentrations of CW (5, 10, 15, and 20%, *v*/*v*). MS basal medium without PGR supplementation and medium supplemented with 0.05 mg/L kinetin and 0.35 mg/L NAA were used as controls. The number and length of the induced roots were determined after 4 weeks of cultivation. One explant was placed in each culture vessel. The experiment was repeated twice, each with ten replicates.

2.5. Ex Vitro Acclimatization of the A. officinalis Plantlets

Four-week-old plantlets of *A. officinalis* cultivated in MS basal medium supplemented with CW were selected and subjected to ex vitro acclimatization. Roots were washed with tap water after being removed from the culture vessel to remove the adhering culture medium. The resulting in vitro plantlets were transplanted in 10.0 cm diameter plastic pots filled with peat moss and vermiculite in a 1:1 ratio. All of the potted plants were transferred to a growth chamber at 25 ± 2 °C with a photoperiod of 16 h, a light intensity of approximately 3000 lux, and a relative humidity of approximately 45–55%. The survival rate and plant growth (plant height) were monitored after 30 days of cultivation. The experiments were performed twice, each with ten replicates.

2.6. Experimental Design and Statistical Analysis

The statistical experimental design, namely, a completely randomized design (CRD), was used in this study with 10 replicates for each treatment. The experimental data were manipulated using analysis of variance (ANOVA) with the SPSS application for Windows. The mean difference between each treatment was determined using Duncan's multiple range test (DMRT) at the 95% confidence level or a probability of $p \leq 0.05$.

3. Results and Discussion

3.1. Effect of Organic Additives on Shoot Proliferation

Several organic additives, such as CW, BH, PH, YE, TJ, PJ, and PP, have been used for plant micropropagation; however, their effects on plant growth and development vary depending on many factors, including plant species and cultivar, plant age, explant type, and cultivation conditions [19,23,24]. In this study, three organic additives, CW, BH, and PH, which are widely used and readily available at low cost, especially in Thailand, were chosen, and their effect on shoot proliferation in *A. officinalis* was evaluated. The effect of CW on shoot proliferation in *A. officinalis* is summarized in Table 1. MS medium without PGR and CW supplementation yielded the lowest number of shoots (1.55 shoots/explant) and average shoot length (2.19 cm). Supplementation of MS medium with 0.05 mg/L NAA and 1.0 mg/L kinetin improved shoot proliferation in *A. officinalis*, leading to an increase in the number of shoots (2.55 shoots/explant) and the average shoot length (3.70 cm). This finding suggests that NAA and kinetin are essential for shoot formation and development, since these PGRs are involved in cell division, differentiation, and elongation [25]. Notably, in the current study, the replacement of kinetin with CW significantly increased the number of shoots and the shoot lengths of *A. officinalis*. Supplementation with CW at concentrations of 15 and 20% (*v*/*v*) yielded the highest values for the number of shoots (4.00–4.10 shoots/explant) and shoot length (7.25–7.26 cm), implying that CW is more effective for promoting shoot formation and development in *A. officinalis* than kinetin at 1.0 mg/L. Apart from natural sources of carbon, mainly sugars, CW also contains some vitamins (thiamin, pyridoxine, ascorbic acid), amino acids, minerals (phosphorus, magnesium, potassium, calcium, iron, and manganese), and PGRs, such as cytokinins, zeatin, auxins (indoleacetic acid, IAA), gibberellic acid (GA), and abscisic acid (ABA), which are essential for plant cell division, differentiation, and development [18,20,24,26,27]. CW has been successfully used to promote shoot proliferation in several plants, such as *Musa acuminata* [24], *Dendrobium chryseum* [28], *Dianthus caryophyllus* [29], *Hylocereus polyrhizus* [30], *Coelogyne pandurata* [21], *Echinacea purpurea* [31], *Cymbidium aloifolium* [32], and *D. cruentum* [22].

Table 1. Effects of coconut water (CW) on shoot proliferation in *A. officinalis* after 4 weeks of cultivation in different MS media.

Medium	Number of Shoots (Shoots/Explant)	Shoot Length (cm)
MS (control)	1.50 ^{c}	2.19 ^{e}
MS + 0.05 mg/L NAA + 1.0 mg/L kinetin	2.60 ^{b}	3.70 ^{d}
MS + 0.05 mg/L NAA + 5% (*v*/*v*) CW	2.60 ^{b}	5.76 ^{c}
MS + 0.05 mg/L NAA + 10% (*v*/*v*) CW	3.40 ^{a}	6.53 ^{b}
MS + 0.05 mg/L NAA + 15% (*v*/*v*) CW	4.00 ^{a}	7.25 ^{a}
MS + 0.05 mg/L NAA + 20% (*v*/*v*) CW	4.10 ^{a}	7.26 ^{a}

According to DMRT analysis, means followed by distinct letters within a column are significantly different at $p \leq 0.05$.

The effect of BH on *A. officinalis* shoot proliferation was investigated, and the results are summarized in Table 2. MS medium without PGR and BH supplementation yielded 1.60 shoots/explant and a shoot length of 2.16 cm. Supplementation of MS medium with NAA and kinetin at 0.05 and 1.0 mg/L promoted shoot proliferation in *A. officinalis*. Similarly, supplementation of the MS medium with BH at 5% (*w*/*v*) also promoted shoot

proliferation compared with MS medium without PGR and BH supplementation, suggesting that BH can compensate for the lack of kinetin. Notably, the number of shoots and the shoot lengths of *A. officinalis* in MS medium supplemented with 5% (*w*/*v*) BH were still lower than those of MS medium supplemented with NAA and kinetin. Furthermore, increasing the concentration of BH in the MS medium over 5% (*w*/*v*) yielded a lower number of shoots and lower shoot lengths, indicating that BH had a negative effect on *A. officinalis* shoot proliferation. Like other organic additives, such as CW or PH, BH contains high amounts of sugars and trace elements, such as potassium, calcium, sodium, iron, manganese, and bromine, as well as vitamins (thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, and ascorbic acid), phenolic acid, carotenoids, and unidentified substances [19,33–35]. These substances are necessary for plant growth and development, and the optimal concentration of each substance depends on plant species, plant age, explant type, and cultivation conditions. For instance, BH at a concentration of 5% (*v*/*v*) was the best for shoot regeneration in *Celosia* sp. [36], while a concentration of 10% (*w*/*v*) was suitable for in vitro regeneration of *Dendrobium* sp. [37]. Another study by Gansau et al. [38] reported that 2.5% (*w*/*v*) BH was effective for shoot and root development during in vitro propagation of *D. lowii*. According to De Stefano et al. [39], BH at a concentration of 1% (*w*/*v*) promoted shoot and root proliferation in *Epidendrum nocturnum*. A recent study by Samala and Thipwong [22] also demonstrated that BH yielded the highest number of in vitro *D. cruentum* plantlets. Notably, the excess of some substances in BH, specifically sugars or some trace elements, such as calcium or sodium, may inhibit cell growth and development. The adverse effects of high concentrations of BH on the growth and development of some plant species have also been reported, e.g., protocorm-like bodies (PLBs) of *Dendrobium* sp. [40] and *Raphanus sativus* [27].

Table 2. Effects of banana homogenate (BH) on shoot proliferation in *A. officinalis* after 4 weeks of cultivation in different MS media.

Medium	Number of Shoots (Shoots/Explant)	Shoot Length (cm)
MS (control)	1.60 ^{bc}	2.16 ^{c}
MS + 0.05 mg/L NAA + 1.0 mg/L kinetin	2.70 ^{a}	3.69 ^{a}
MS + 0.05 mg/L NAA + 5% (*w*/*v*) BH	2.00 ^{b}	3.08 ^{a}
MS + 0.05 mg/L NAA + 10% (*w*/*v*) BH	1.30 ^{c}	1.55 ^{d}
MS + 0.05 mg/L NAA + 15% (*w*/*v*) BH	0.60 ^{d}	0.31 ^{e}
MS + 0.05 mg/L NAA + 20% (*w*/*v*) BH	0.20 ^{d}	0.06 ^{e}

Means followed by different letters within a column are significantly different at $p \leq 0.05$ based on DMRT analysis.

Considering the effect of PH on *A. officinalis* shoot proliferation, supplementation with this organic additive promoted shoot formation and shoot length compared to MS medium with and without PGR supplementation (Table 3). The maximum number of shoots (4.00 shoots/explant) and shoot length (7.22 cm) of *A. officinalis* were determined in MS medium supplemented with 0.05 mg/L NAA and 10% (*w*/*v*) PH. Higher concentrations of PH at 15 and 20% (*w*/*v*) reduced the number of shoots and the shoot lengths, indicating that this organic additive had a negative effect on plant cell growth and development, possibly due to an oversupply of the trace elements, such as potassium, iron, or magnesium, contained in the PH, as reported in other studies. As previously reported, PH contains several organic and inorganic substances, such as carbohydrates, amino acids, vitamins (thiamin, pyridoxine, and ascorbic acid), minerals (potassium, iron, and magnesium), PGRs, and unidentified substances that are involved in cell growth, differentiation, and development [41,42]. This organic additive has been widely used for the in vitro propagation of several plants, and the response of plant cells to PH depends on plant species and explant types. For example, Ouyang et al. [43] reported that PH at 5% (*w*/*v*) promoted callus formation and green shoot regeneration in *Triticum aestivum*. However, a higher concentration of this organic additive resulted in reduced green shoot regeneration. According to a study

by Islam et al. [44], 100 mL/L PH promoted seedling growth for in vitro cultures of *Vanda roxburgii*. Increasing the concentration of PH led to a reduction in seedling growth in this orchid. A recent study by Rohmah et al. [32] demonstrated that supplementing MS medium with 10 g/L PH and 100 mL/L CW or 5 g/L PH and 150 mL/L CW yielded the highest number of shoots in *C. aloifolium*, while increasing the concentration of PH yielded a lower number of shoots.

Table 3. Effects of potato homogenate (PH) on shoot proliferation in *A. officinalis* after 4 weeks of cultivation in different MS media.

Medium	Number of Shoots (Shoots/Explant)	Shoot Length (cm)
MS (control)	1.60 ^{c}	2.17 ^{e}
MS + 0.05 mg/L NAA + 1.0 mg/L kinetin	2.50 ^{b}	3.67 ^{d}
MS + 0.05 mg/L NAA + 5% (*w*/*v*) PH	2.70 ^{b}	5.27 ^{c}
MS + 0.05 mg/L NAA + 10% (*w*/*v*) PH	4.00 ^{a}	7.22 ^{a}
MS + 0.05 mg/L NAA + 15% (*w*/*v*) PH	3.80 ^{a}	6.23 ^{b}
MS + 0.05 mg/L NAA + 20% (*w*/*v*) PH	3.70 ^{a}	6.29 ^{b}

Means followed by different letters within a column are significantly different at $p \leq 0.05$ based on DMRT analysis.

Among the different types of organic additives tested in this study, CW exhibited superior performance in promoting *A. officinalis* shoot proliferation, followed by PH and BH. These organic additives contain several chemical constituents, including carbohydrates, vitamins, amino acids, trace elements, and PGRs [18–20,24,26,27,33–35,41,42]. The outperforming of CW for promoting shoot proliferation in *A. officinalis* could be attributed to the types of carbohydrates found in this material. The prime carbohydrates found in CW are soluble carbohydrate compounds, specifically sucrose, glucose, and fructose, which are efficiently taken up by plant cells, while those contained in BH and PH are starches, which have to be broken down into simple sugars before being taken up by plant cells. Another possibility is that CW is rich in PGRs, especially auxin and cytokinin, as well as vitamins, compared to BH and PH. Adding CW to the culture medium may provide sufficient phytohormones and vitamins to accelerate plant cell growth, differentiation, and development [25,45].

It should be noted in the current study that the number of shoots and the shoot lengths of *A. officinalis* cultivated in MS medium supplemented with CW and PH were nearly identical. However, adding PH to the MS medium caused the young plantlets to turn yellow, while CW produced greener and healthier plants. Therefore, CW was chosen to further investigate its effect on root proliferation in *A. officinalis*.

3.2. Effect of Coconut Water on Root Proliferation

In addition to sugars, vitamins, amino acids, organic acids, and minerals, CW is also rich in phytohormones, specifically natural auxins, which play an essential role in all plant physiological processes, including the induction of adventitious and lateral roots [25]. The major auxin predominantly found in CW is indole acetic acid (IAA) [45]. Based on the literature review, CW has been successfully utilized as a growth supplement for in vitro and ex vitro root proliferation in several plants, such as kiwifruit (*Actinidia deliciosa*) [46], mangrove (*Rhizopora stylosa*) [47], Tribulus (*Tribulus terrestris*) [48], and banana (*Musa* cv. Rajabulu) [49]. Notably, the application of CW for root induction in asparagus has never been documented. Therefore, the effect of CW as a growth supplement for in vitro root proliferation in *A. officinalis* was investigated in the current study. As shown in Table 4, the formation of *A. officinalis* roots was also observed in the control treatment, yielding 2.50 roots/explant with a root length of 1.55 cm. This finding suggested that this plant rooted easily in MS medium without PGR supplementation, similar to other plants, such as *Philodendron* sp. [50]. MS medium supplemented with 0.35 mg/L NAA and 0.05 mg/L kinetin yielded a higher number of roots (3.10 roots/explant) than the control

MS medium, while root length was not significantly different from the control, indicating that a combination of auxins and cytokinins could enhance *A. officinalis* root formation, consistent with the findings of Desjardins et al. [51], Shigeta et al. [52], and Rasad et al. [14]. It has also been reported that auxins alone can promote root formation in *A. officinalis*. For instance, Maung et al. [15] showed that 2.5 mg/L IBA resulted in the highest rooting rate of 44%, while Minh et al. [16] discovered that NAA at a concentration of 0.5 mg/L substantially improved the rooting rate by up to 74.59%.

Table 4. Effect of coconut water (CW) on root proliferation in *A. officinalis* after 4 weeks of cultivation in different MS media.

Medium	Number of Roots (Roots/Explant)	Root Length (cm)
MS (control)	2.50 ^{ab}	1.55 ^{c}
MS + 0.35 mg/L NAA + 0.05 mg/L kinetin	3.10 ^{a}	1.33 ^{c}
MS + 5% (*v*/*v*) CW	1.80 ^{c}	4.54 ^{b}
MS + 10% (*v*/*v*) CW	2.30 ^{bc}	4.88 ^{b}
MS + 15% (*v*/*v*) CW	2.60 ^{ab}	5.35 ^{a}
MS + 20% (*v*/*v*) CW	3.20 ^{a}	5.50 ^{a}

Means followed by different letters within a column are significantly different at $p \leq 0.05$ based on DMRT analysis.

Considering the effect of CW on the in vitro rooting of *A. officinalis*, the concentrations of 5% and 10% (*v*/*v*) CW did not significantly induce root formation in the plant, but instead promoted root elongation, yielding approximately root lengths of 4.54 and 4.88 cm, respectively. Increasing the concentrations of CW to 15 and 20% (*v*/*v*) significantly improved *A. officinalis* root proliferation, suggesting that CW at these concentrations is suitable for root formation in this plant, possibly due to the hormonal balance between auxins and cytokinins, which could promote root formation in the explants [25]. The maximum number of roots (3.20 roots/explant) and root lengths (5.50 cm) were observed in the MS medium supplemented with 20% (*v*/*v*) CW. These results are consistent with the findings reported for other plants, such as *Daphne* sp. [53], *R. stylosa* [47], and *Epidendrum nocturnum* [39]. Based on the results obtained in this study, CW can be utilized as an alternative root-setting medium instead of using commercial rooting hormones for the in vitro propagation of *A. officinalis*.

3.3. Ex Vitro Acclimatization of In Vitro Regenerated A. officinalis

Like other in vitro propagated plants, an ex vitro acclimatization process is required to ensure plant growth and survival of well-developed in vitro *A. officinalis* plantlets when transplanted to soil or field environments. A variety of supporting materials or rooting substrates have been utilized as planting media for ex vitro acclimatization, such as sand, coco peat, rockwool, rice husk, peat moss, vermiculite, and perlite [54–57]. Among these materials, peat moss and vermiculite are the most commonly used, since they have a higher water-holding capacity than the others. Furthermore, peat moss is also rich in nutrients, which could promote plant growth and development [57–59]. The ex vitro acclimatization of micropropagated *A. officinalis* was investigated in this study by using peat moss and vermiculite at a ratio of 1:1 as supporting materials. As shown in Table 5 and Figure 1, all of the in vitro plantlets were successfully acclimatized with 100% survival rates. Furthermore, the acclimatized plants also exhibited morphology comparable to that of the mother plants. The in vitro plantlets from the control treatment or the MS-free hormone produced the lowest number of shoots and roots compared to those from other treatments. However, the plantlets from this control medium exhibited relatively longer shoot and root lengths than those from MS medium supplemented with 0.35 mg/L NAA and 0.05 mg/L kinetin. Notably, the plantlets from MS medium supplemented with CW exhibited significantly higher numbers of shoots and roots as well as longer shoot and root lengths than those from the MS-free hormone, particularly in the medium with high concentrations of CW.

Among different concentrations of CW, the plantlets from the medium supplemented with 20% (*v*/*v*) CW yielded the highest values for the number of shoots (6.60 shoots/explants), shoot length (12.79 cm), number of roots (4.80 roots/explant), and root length (6.03 cm). These results demonstrated that CW not only promotes in vitro plant propagation but also accelerates ex vitro plant growth and development, in agreement with findings reported for *Dracaena purplecompacta* [60] and *R. stylosa* [47].

Table 5. The growth of *A. officinalis* after 4 weeks of acclimatization using peat moss and vermiculite at a ratio of 1:1 as supporting material.

In Vitro Plantlet from the Medium	Number of Shoots (Shoots/Plantlet)	Shoot Length (cm)	Number of Roots (Roots/Explant)	Root Length (cm)
MS (control)	2.30 ^{c}	11.15 ^{c}	2.90 ^{d}	2.74 ^{b}
MS + 0.35 mg/L NAA + 0.05 mg/L kinetin	6.30 ^{a}	9.23 ^{e}	4.10 ^{bc}	2.27 ^{c}
MS + 5% (*v*/*v*) CW	4.80 ^{b}	10.62 ^{d}	3.70 ^{c}	5.73 ^{a}
MS + 10% (*v*/*v*) CW	5.80 ^{a}	11.66 ^{b}	4.50 ^{ab}	5.75 ^{a}
MS + 15% (*v*/*v*) CW	6.50 ^{a}	12.56 ^{a}	4.70 ^{ab}	5.92 ^{a}
MS + 20% (*v*/*v*) CW	6.60 ^{a}	12.79 ^{a}	4.80 ^{a}	6.03 ^{a}

Means followed by different letters within a column are significantly different at $p \leq 0.05$ based on DMRT analysis.

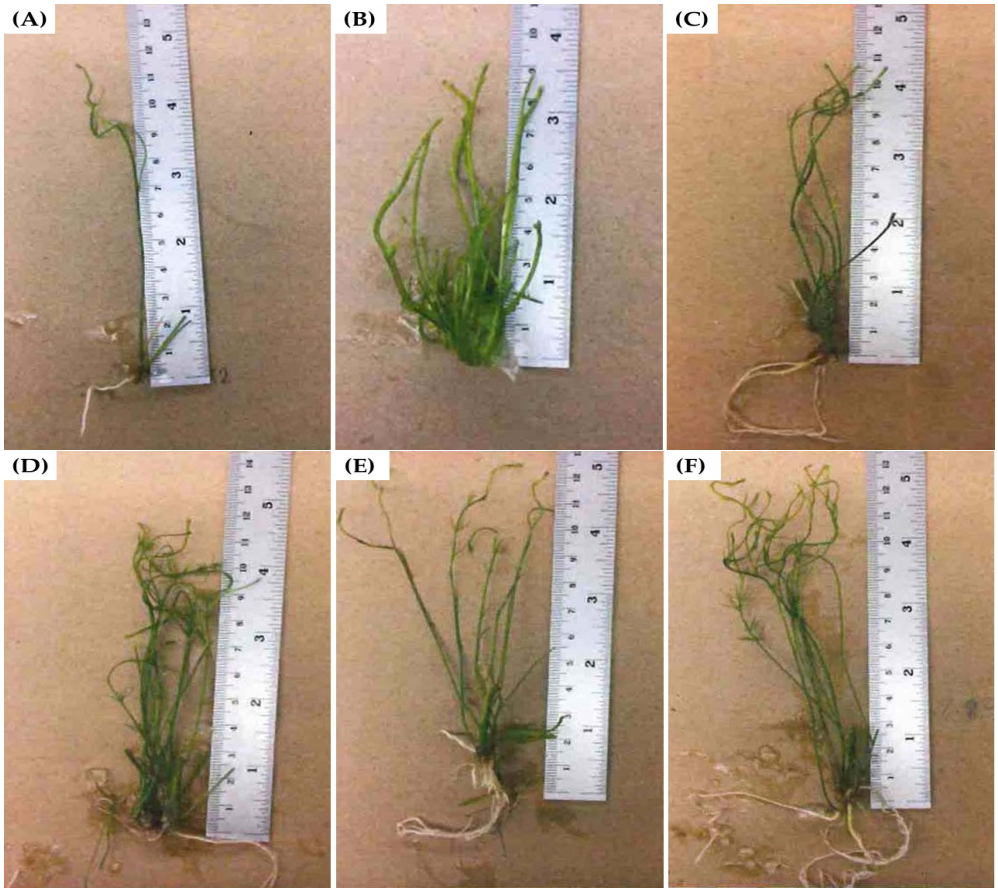

Figure 1. The plantlets of *A. officinalis* after ex vitro acclimatization for 4 weeks using peat moss and vermiculite at a ratio of 1:1 as supporting materials. The in vitro plantlets grown from MS-free hormone (**A**), MS medium supplemented with 0.35 mg/L NAA and 0.05 mg/L kinetin (**B**), MS medium supplemented with 5% (*v*/*v*) CW (**C**), MS medium supplemented with 10% (*v*/*v*) CW (**D**), MS medium supplemented with 15% (*v*/*v*) CW (**E**), and MS medium supplemented with 20% (*v*/*v*) CW (**F**).

The present results clearly demonstrated that CW is a promising material that can be used as a growth supplement for the micropropagation of *A. officinalis*. In addition to being a natural carbon source, it is also rich in natural vitamins, amino acids, minerals, and phytohormones, which play an essential role in plant growth and development. Furthermore, CW is cheap, readily available, and easy to prepare and apply to the plant cultivation medium, promoting the sustainability of the commercial propagation of *A. officinalis*.

Although the regenerated plants revealed morphological traits similar to those of the mother plant (Figure 1), the in vitro culture environment can trigger mutations in the new plantlets, known as somaclonal variations [18]. Identifying somaclonal variations is crucial in in vitro propagating plants. Several techniques have been employed; among these, PCR-based molecular markers, such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), and start codon targeted (SCoT) polymorphism markers, are widely used [18]. Therefore, further investigation of their genetic stability is needed to verify the new plantlets of *A. officinalis*.

4. Conclusions

As demonstrated in this study, organic additives, including CW, BH, and PH, could be used as growth-promoting materials for the in vitro propagation of *A. officinalis*. Among these organic additives, CW exhibited great potential for promoting shoot and root proliferation in *A. officinalis* compared to BH and PH. Supplementation of the MS basal medium with 20% (*v*/*v*) CW yielded the highest number of shoots (4.10 shoots/explant) and roots (3.20 roots/explant) and the longest shoots (7.26 cm) and roots (5.50 cm); these values were higher than those obtained from MS-free hormone and MS supplemented with NAA and kinetin. Furthermore, the in vitro plantlets regenerated from the MS medium supplemented with CW, especially at 20% (*v*/*v*), also exhibited higher growth performance than those regenerated using other treatments, with morphological characteristics similar to those of the mother plant. This report is the first to demonstrate that organic additives, specifically CW, are the best growth supplement material for the in vitro propagation of *A. officinalis* and can be applied instead of commercial phytohormones to minimize the costs of large-scale plant production.

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