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Survival Rate, Chemical and Microbial Properties of Oak Seedlings Planted with or without Oak Forest Soils in a Black Locust Forest of a Dryland

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Abstract: Native tree species are frequently unable to effectively grow in non-native tree cultivation scenarios. In the Loess Plateau, China, it is difficult to find native oak (*Quercus liaotungensis*) seedlings in non-native black locust forests. Black locust is an arbuscular mycorrhizal (AM) tree, but oak is an ectomycorrhizal (ECM) tree. Plants highly depend on their symbiotic mycorrhizal fungi to take up water, nitrogen (N) and other nutrients. We hypothesized that black locust forests would not provide ECM inoculum to oak seedlings, limiting their water and nutrient uptake, which would be improved by ECM inoculum. Here, we (1) sowed seeds, with or without oak forest soils, (2) transplanted seedlings collected in oak forests, with or without oak forest soils, and (3) planted seedlings germinated in autoclaved or unautoclaved oak forest soils. We measured the survival and growth rate for all three experiments, along with chemical properties, and root ECM colonization. Oak seeds sowed with oak forest soils had higher mycorrhizal colonization, leaf N concentrations and survival rate, and lower root $\delta^{13}C$ than the seeds sowed without oak forest soils. Planting with oak forest soils also increased the survival rate of the germinated seedlings, but not the transplanted seedlings. Overall, our study suggested that the use of oak forest soils in the black locust forest to improve the water and N uptake of oak seedlings by providing the ECM inoculum, resulting in a high survival rate. Our study also implies that the method of sowing seeds was effortless and effectively compared to transplanting wild/nursery seedlings.

Keywords: exotic tree; ectomycorrhizal fungi; forest mycorrhizal type; succession

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1. Introduction

There are many reports around the world of forests dominated by non-native trees due to either plantation or invasion, and the replacement of native trees is needed for ecological balance [1–3]. However, in many cases, native tree species cannot grow well under non-native cultivations [4–6]. It is necessary to have native trees able to grow in conjunction with non-native trees, in order to allow the forests to undergo a smooth and successful transition back to native forests. There are many studies which have investigated the reasons behind the inability of native tree species to grow in non-native tree plantations, but the reasons for slow succession are various, making management difficult. The delayed succession has been attributed to things such as the allelopathic effect [1,7], an increase in pathogens [2],

and a disruption of belowground mutualism [3]. To achieve a better understanding of these implications, more case studies are needed to investigate the chemical and microbial properties of seedlings and soils. Additionally, since natural succession is sometimes very slow, especially in drylands [8,9], making it an inefficient, difficult, and expensive management strategy in some countries, it is important to develop an easy way to grow these native seedlings which incorporates the understanding of the chemical and microbial properties of the seedlings.

Soil inoculation is one of the most efficient ways to improve seedling growth, partly through providing mycorrhizal fungi to the seedlings [10,11]. The symbiosis of seedlings with mycorrhizal fungi have significant impacts on their growth and survival rate [12,13]. Accordingly, it has been reported that ectomycorrhizal (ECM) trees are less successfully established than arbuscular mycorrhizal (AM) trees in AM forests [14], and also ECM trees exhibit higher growth rate in AM than ECM forests [15]. These studies reported that it is important for ECM tree seedlings to be provided with ECM fungi that come directly from ECM forest soils [14,15]. Mycorrhizal fungi is known to help trees uptake water, nitrogen (N) and phosphorus, which is necessary for tree growth and survival [16–18]. Providing cultured mycorrhiza to seedlings usually improved the survival rate [19,20]. However, pre-inoculation with the cultured fungi requires effort and funding, thus, easier methods, such as pre-inoculating simply with inoculative soils, or directly sow/planting seeds/seedlings with inoculative soils should be tested in field experiments.

In the Loess Plateau in China, non-native black locust trees have been substantially planted in recent years, although the native climax tree in this region is the oak (*Quercus liaotungensis*) [21]. In the black locust forest, plant diversity was low compared to the oak forest [22], and natural succession to the oak forest has not been observed to date. We hypothesized that the black locust trees would not provide a suitable mycorrhizal inoculum source for oak seedlings, because the black locust is an AM tree and the oak is an ECM tree [23,24]. Here, we examined the ability of the inoculum of ECM fungi with ECM forest soils to improve the growth and survival rate of oak seedlings in the black locust forest. To this end, we sow/planted oak seeds/seedlings in the black locust forest in three easy and cost-effective ways. We (1) sowed seeds with or without oak forest soils, (2) planted seedlings collected in oak forests, with or without oak forest soils, and (3) planted seedlings germinated in autoclaved or unautoclaved oak forest soils. We measured the survival and growth rate, chemical properties, and root ECM colonization. We also collected wild oak seedlings in oak and black locust forests, though these are rare, and we measured the same properties as above in order to know the basic condition of surviving seedlings during the natural regeneration process. Our hypothesis is that ECM inoculum improves the growth and chemical properties of seedlings. Our study will contribute to understanding an efficient way to grow native oak seedlings in the non-native black locust forest.

2. Materials and Methods

2.1. Study Site

This study was conducted in a black locust (*Robinia pseudoacacia*) forest and an oak (*Quercus liaotungensis*) forest in the central part of the Loess Plateau of China. The study site was located near Yan'an city (Mt. Gonglushan, 36°25′ N, 109°32′ E) in the Shaanxi province, China. The vegetation type was categorized as a forest-steppe transitional zone [25]. Mean annual precipitation and mean annual air temperature were 514 mm and 10.2 °C, respectively. This area experiences hot summers and cold winters, with heavy rainfall in the summer [22], and thus plants can relatively easily acquire water resources during the growing season. Both forest canopies were closed, and more than 90% of the canopy was occupied by the dominant species, black locust or oak. Both forests had understory coverage that consisted of shrubs and herbaceous species [26]. At each forest, four plots (20 m × 20 m) which were more than 30 m away from other plots were established. All the plots were located on a flat or gentle slope near the ridge. More detailed information

about forest properties is available in [26]. The relative photosynthetic photon flux density (rPPFD) at approximately 50 cm above the ground of the black locust and oak forest are 7.6 ± 2.7% and 9.7 ± 3.2%, respectively [26]. The representative ECM fungi in this oak forest are Thelephoraceae, Pezizaceae, and Sebacinaceae members [24].

2.2. Field Experiemnt

We conducted a sow/planting experiment of oak seedlings in the black locust forest using three types of experiments to determine the best means of sow/planting the native species. When establishing the subplots, we tried to make the paired subplots show the same rPPFD values.

2.2.1. Experiment 1: Sowing Seeds

A pair of subplots were established in each of the three plots (six subplots in total) of the black locust forest in September 2015. More than 1512 oak seeds and soils were collected in the oak forest. One hundred and ninety-six seeds were buried with the oak forest soil in one subplot (1.5 m × 1.5 m) and 196 seeds were buried without oak forest soils in the other subplot (<5 m away from the other subplot) in each plot.

2.2.2. Experiment 2: Transplanting Wild Seedlings

An additional pair of subplots were established in each of the three plots (six subplots in total) of the black locust forest in June 2015. More than 180 healthy 1-year-old oak seedlings with their whole root systems and soils were collected in the oak forest in June 2015. Thirty seedlings were planted with approximately 50 g of oak forest soil in one subplot (30 cm × 30 cm) and 30 seedlings were planted without oak forest soils in the other subplot (<5 m away from the other subplot) in each plot. We selected similar sized seedlings for this experiment (around 10 cm height). In addition, we did not use the seedlings with tap roots which appeared to be cut or damaged.

2.2.3. Experiment 3: Transplanting Inoculated Seedlings

More than 400 oak seeds collected in September 2015 were rinsed in tap water for one night, and sterilized using 10% H_2O_2 [27], soaked in clean water for one day, and then washed with sterilized pure water. A portion of oak forest soils was autoclaved at 120 °C for 1 h. Half of the sterilized oak seeds were germinated in the unautoclaved oak forest soils to inoculate them with mycorrhiza, and the other half were germinated in the autoclaved oak forest soils to avoid mycorrhizal inoculation, from September 2015 to April 2016, using plastic bags with small holes for ventilation. A pair of subplots were established in each of the four plots (eight subplots in total) of the black locust forest in April 2016. Forty-two seedlings that were germinated in the unautoclaved soils were planted in one subplot (1 m × 1 m), and 42 seedlings germinated in the autoclaved soils, which also observed to have mycorrhiza in their roots, were planted in the other subplot (<5 m away from the other subplot) in each plot. Autoclaved soils are generally used to germinate control seedlings without ECM colonization [28]. There was no visible ECM colonization in seedlings germinated in autoclaved soils, although there was some visible ECM colonization in seedlings germinated in unautoclaved soils.

The shoot lengths of the aboveground seedlings were measured for the subplots used in Experiments 1–3 in June 2016 and September 2016. It was difficult to collect the shoot length data in June 2018 because of the limited number of surviving seedlings. The survival rates of the seedlings were recorded in June 2016, September 2016, and June 2018. The survival rate (%) was calculated based on the number of sowed seeds (196 for Experiment 1), planted seedlings (30 for Experiment 2), and transplanted seedlings (42 for Experiment 3). This could be used as the germination rate for June and September 2016 in Experiment 1, but it was not clear whether all the germinated seedlings survived until the observed date (June and September 2016). Since the number of seedlings in the oak-forest-soil subplot was higher than the black locust-forest-soil subplot, we were not able

to collect all of the seedlings in the oak-forest-soil subplots (more samples were collected than in the black locust-forest-soil subplots) in Experiment 1. The seedlings were separated into leaves and roots, and the weight, length, and diameter of the shoots and roots were measured. The seedlings were carefully washed with pure water. Twelve seedlings from both the oak-forest-soil and black locust-forest-soil subplots (total 24 seedlings) were frozen for quantifying mycorrhizal colonization. The rest of the seedlings (9 seedlings form each subplot) were oven-dried at 60 °C for over 24 h and kept for further chemical analysis.

2.3. The Collection of Wild Seedling Samples

Six and eighteen oak seedlings were found and collected in the black locust forest in June 2016 and in August 2017, respectively, and the same or slightly more oak seedlings were collected in the oak forest at the same time. It was difficult to find the oak seedlings in the black locust forest, resulting in the limited number of seedling samples, although we were looking for the seedlings for over 2 h, with multiple people, in a plot that measured 100 m × 30 m. The seedlings from June 2016 were separated into leaves and roots, and the weight, length, and diameter of shoots and roots were measured. All samples were carefully washed with pure water, and then oven-dried at 60 °C for over 24 h and kept for further chemical analysis. Most of the seedlings were 1 year old, and all seedlings were ≤2 years old.

2.4. Laboratory Analysis

Leaf and root samples (from experiments 1, and the wild seedling sampling) were ground and loaded into capsules for isotope analysis. Total N and C concentration and stable isotope ratios were measured using a mass spectrometer (Deltaplus XP, Thermo Electron, Waltham, MA, USA) with an elemental analyzer (Flash EA 1112, Thermo Electron, Germany). The precision of the procedure was better than ± 0.2‰ for the isotope ratio. Natural abundances of ^{15}N and ^{13}C were expressed in per mil (‰) deviation from international standards:

$$\delta^{15}N \text{ or } \delta^{13}C = [R_{sample}/R_{standard} - 1] \times 1000,$$

where R is $^{15}N/^{14}N$ or $^{13}C/^{12}C$, respectively. Atmospheric N, Pee Dee Belemnite were used as the international standards for N and C, respectively.

2.5. Statistical Analysis

For the size and chemical properties of the grown seedlings in Experiment 1, an analysis of variance (ANOVA) for a linear mixed model (LMM) was used to test the significant differences between the treatment based on soils used to grow the seedlings (with/without oak forest soils in Experiment 1 and 2, autoclaved/unautoclaved oak forest soils in Experiment 3). For the survival rate (or germination/survival rate of Experiment 1 in June and September 2016) of the seedlings in each experiment, we used ANOVA for a generalized linear mixed model (GLMM) using the binomial family to test the significant differences between the treatment and recoding occasion. For the aboveground length of seedlings in each experiment, we used ANOVA for LMM to test the significant differences between the treatment and recoding occasions. The difference in the plots was set as a random effect in these tests. For the leaf C and N of wild seedlings collected on different occasions, the ANOVA for linear model (LM) was used to test the significant differences between the two forests and recording occasions. For the size and other chemical properties of wild seedlings that were collected on one occasion, ANOVA for LM was used to test the significant differences between the two forests. The lme4, lmerTest, and car packages [29–31] in R (version 4.0.0; [32]) were used for the models. We defined a significance level of $p < 0.05$ for all tests.

3. Results

3.1. Planting Experiment

3.1.1. Experiment 1: Sowing Seeds

The germination/survival rate of the seedlings was significantly higher (F = 18.2, $p < 0.001$) with oak forest soils (Figure 1). The germination/survival rate was not the highest shortly after sowing because we could only count individuals which appeared aboveground, but some seedlings may have been alive belowground without stems and leaves. The aboveground height was significantly shorter (F = 4.2, p = 0.040) with the oak forest soils than without (Figure 1). The aboveground and belowground N concentration and C:N ratio were significantly different between the seedlings grown with different soil origin (Table 1). Both N concentrations were higher, and both C:N ratios were lower in the seedlings grown with oak forest soils. The N concentrations were ca. 1.0% in the black locust forest and ca. 2.5% in the oak forest, and the C:N ratios were ca. 44% in the black locust forest and ca. 18% in the oak forest. Belowground $\delta^{13}C$ and the percentage of mycorrhizal tips also significantly differed between the soil origin. The belowground $\delta^{13}C$ was lower and the percentage of mycorrhizal tips were higher in the seedlings grown with the oak forest soils (Table 1).

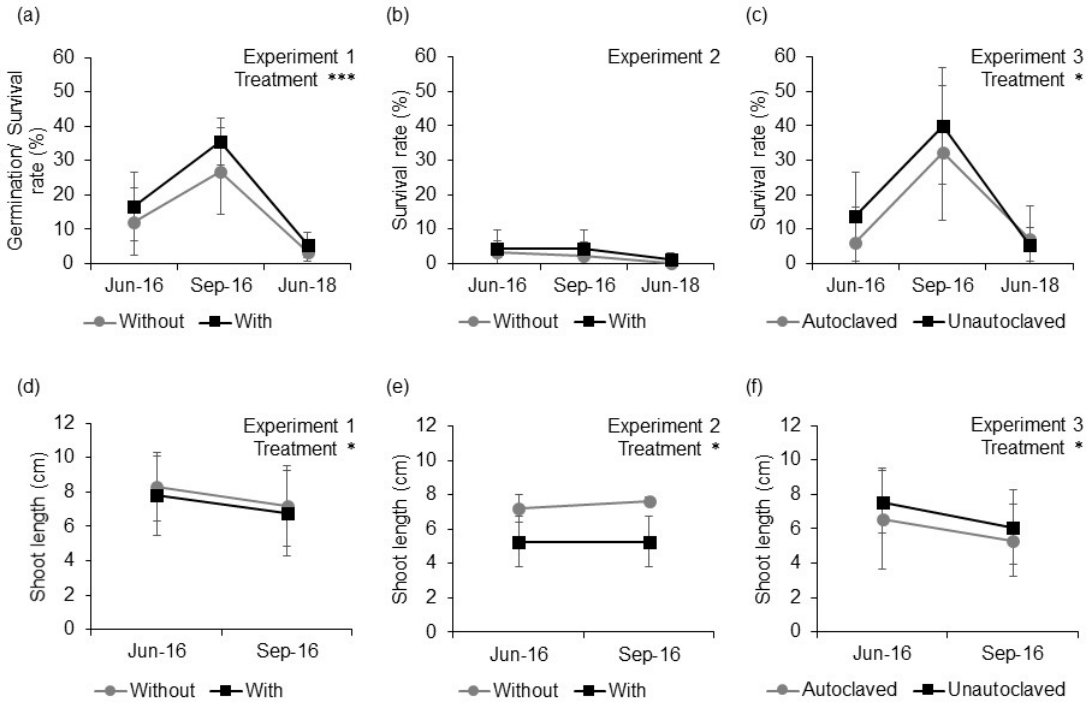

Figure 1. The germination/survival rates (**a**), survival rates (**b**,**c**), and the aboveground height (**d**–**f**) of the sow/planted seedlings. Values are means ± SD. The asterisk on "Treatment" means p-values (* $p < 0.05$, *** $p < 0.001$) based on ANOVA for the GLMM (**a**–**c**) or LMM (**d**–**f**) with the treatment (with/without oak forest soils in Experiment 1 and 2, autoclaved/unautoclaved oak forest soils in Experiment 3) and recording occasion. The models were performed with the plot as a random variable. (**a**,**d**) Differences between sowing seeds with or without oak forest soils in Experiment 1, (**b**,**e**) differences in transplanting wild seedlings with or without oak forest soils in Experiment 2, (**c**,**f**) differences in transplanting seedlings inoculated in autoclaved or unautoclaved oak forest soils in Experiment 3.

Table 1. The size and chemical properties (least square mean ± SE) of the sowed seedlings of June 2018 under Experiment 1 (sowing seeds) in the black locust forest without and with the oak forest soils. (*n*) shows the number of samples. The right side shows *F*-values and *p*-values (*** $p < 0.001$) based on ANOVA for the LMM with the treatment (with or without the oak forest soils). The models were performed with the plot as a random variable. The significant results were bolded.

Sample	Measurement	Treatment				*F* Value	
		without Oak Forest Soils	(*n*)	with Oak Forest Soils	(*n*)		
Aboveground	Weight (mg)	116 ± 33	(21)	129 ± 32	(21)	0.2	
	Length (cm)	6.57 ± 0.83	(21)	7.01 ± 0.82	(21)	0.5	
	Diameter (mm)	1.34 ± 0.23	(21)	1.29 ± 0.23	(21)	0.1	
	C concentration (%)	41.7 ± 0.65	(9)	43.3 ± 0.64	(9)	3.8	
	N concentration (%)	1.00± 0.22	(9)	2.52 ± 0.22	(9)	**34.2**	***
	C:N ratio	44.2 ± 4.0	(9)	18.2 ± 3.9	(9)	**28.5**	***
	δ^{13}C (‰)	−28.2 ± 0.8	(9)	−29.0 ± 0.8	(9)	2.0	
	δ^{15}N (‰)	−3.2 ± 0.3	(9)	−2.7 ± 0.3	(9)	4.4	
Belowground	Weight (mg)	352 ± 74	(21)	456 ± 73	(21)	2.1	
	Length (cm)	15.8 ± 1.54	(21)	19.4 ± 1.46	(21)	3.1	
	Diameter (mm)	3.02 ± 0.21	(21)	3.05 ± 0.21	(21)	0.0	
	C concentration (%)	42.5 ± 0.57	(9)	43.7 ± 0.57	(9)	3.1	
	N concentration (%)	1.03 ± 0.18	(9)	2.53 ± 0.18	(9)	**43.8**	***
	C:N ratio	45.4 ± 5.14	(9)	20.3 ± 5.28	(9)	**28.9**	***
	δ^{13}C (‰)	−27.6 ± 0.4	(9)	−30.0 ± 0.4	(9)	**32.4**	***
	δ^{15}N (‰)	−3.4 ± 0.4	(9)	−2.9 ± 0.4	(9)	1.0	
	Root tips	148 ± 53.1	(12)	220 ± 53.9	(12)	1.0	
	Mycorrhizal tips (%)	7.92 ± 1.71	(12)	18.90 ± 1.73	(12)	**23.5**	***

3.1.2. Experiment 2: Transplanting Wild Seedlings

The survival rate was not different ($F = 1.3$, $p = 0.259$) between the soil origin, while the aboveground height was significantly shorter ($F = 9.8$, $p = 0.011$) than without oak forest soils (Figure 1).

3.1.3. Experiment 3: Transplanting Inoculated Seedlings

Survival rate ($F = 4.1$, $p = 0.042$) and the aboveground height ($F = 5.6$, $p = 0.020$) of the seedlings was significantly higher with unautoclaved oak forest soils (Figure 1).

3.2. Wild Seedlings

The aboveground and belowground C:N ratio and the δ^{13}C and δ^{15}N significantly differed between the wild seedlings from the two forests (Table 2). The δ^{13}C and δ^{15}N was higher in the black locust forest, and the C:N ratio was higher in the oak forest. The belowground N concentration also significantly differed between the seedlings from the two forests and was higher in the black locust forest (Table 2).

Table 2. Size and chemical properties (mean ± SE) of wild seedlings collected in the black locust forest and in the oak forest. (*n*) shows the number of samples. On the right are *F*-values and *p*-values (** $p < 0.01$, *** $p < 0.001$) based on the ANOVA for LM with the forest (the black locust forest or the oak forest). For the leaf C and N of wild seedlings collected on different occasions, LM for the forest type and recording occasion was used. For the size and other chemical properties of wild seedlings collected on one occasion, LM for forest type was used. The significant results were bolded.

Sample	Measurement	Black Locust Forest	(*n*)	Oak Forest	(*n*)	*F* Value	
Aboveground	C concentration (%)	44.8 ± 0.9	(18)	45.5 ± 0.5	(20)	1.4	
	N concentration (%)	1.65 ± 0.11	(18)	1.43 ± 0.18	(20)	3.5	
	C:N ratio	27.7 ± 1.6	(18)	34.1 ± 3.4	(20)	**9.0**	**
	δ^{13}C (‰)	−24.7 ± 0.33	(6)	−28.4 ± 0.12	(6)	**112.1**	***
	δ^{15}N (‰)	−3.42 ± 0.28	(6)	−6.50 ± 0.77	(6)	**14.3**	**
Belowground	C concentration (%)	42.9 ± 0.6	(6)	42.7 ± 0.4	(6)	0.2	
	N concentration (%)	1.17 ± 0.11	(6)	0.66 ± 0.06	(6)	**16.8**	**
	C:N ratio	38.5 ± 4.0	(6)	66.7 ± 5.9	(6)	**15.6**	**
	δ^{13}C(‰)	−24.1 ± 0.4	(6)	−27.4 ± 0.2	(6)	**63.0**	***
	δ^{15}N (‰)	−3.31 ± 0.26	(6)	−5.35 ± 0.31	(6)	**25.2**	***

4. Discussion

In agreement with our expectation, the survival of the oak seedlings sow/planted with the oak forest soils was significant compared with the seedlings sow/planted without oak forest soils (Figure 1). This is consistent with other reports that found soil inoculation improved the seedling growth [10,11]. In addition, our study showed that the seeds had almost the same survival rate as the inoculated seedlings that were transplanted. The timing of the planting of the seedlings in Experiment 2 (transplant of wild seedlings) could have affected the survival rate; seedlings were not planted in the best season, as early spring is typically better for the planting of seedlings. In addition, in our study, the mortality of the inoculated and transplanted seedlings in Experiment 3 was high compared with that in other studies [33,34], probably because of high drought stress typical to drylands, but we could not determine the exact reason in this study. Planting techniques using plastic to reduce drainage and evaporation may improve the survival rate although they are expensive [35]. The selection of ECM fungal species for inoculation may also increase survival and growth [33,36]. Overall, we recommend sowing oak seeds with oak forest soils to increase their survival rate and the method is easy to implement (Figure 1).

The seedlings grown with oak forest soils had a higher mycorrhizal colonization rate, leaf N concentration, and lower root δ^{13}C value (Table 1). Water stress causes stomatal closure, which leads to lower fractionation during photosynthesis, resulting in high foliar δ^{13}C [37,38]. It has been reported that the effect of water stress on foliar and root δ^{13}C were consistent [39]. Thus, the higher root δ^{13}C of seedlings grown without oak forest may suggest they were under high water stress for a longer time than seedlings grown with oak forest soil, although the foliar δ^{13}C was not significantly but slightly higher in the seedlings with (as opposed to without) oak forest soils (Table 1). Wild seedlings in the black locust forest would also suffer from water stress compared to the seedlings living in the oak forest (Table 2). The ECM fungal inoculation would help seedlings to acquire more water and N as frequently reported [40–42]. Drought severely reduces the survival rate of seedlings [43,44], and plant water condition and leaf N concentration are other important factors controlling the photosynthetic rate [45–47], which may contribute to high survival rate with oak forest soils. We also found some ECM colonization in the seedlings grown without oak forest soils (Table 1), which implies that our method to sterilize seeds (10% H_2O_2) was not optimal, as 30% H_2O_2 was recommended in a previous study [48]. However, it is apparent that oak forest soils largely improved the survival rate of oak seedlings, as described above.

Introduced exotic ECM trees were reported to share ECM fungi with native ECM trees [49,50], but the black locust is an AM tree and could not provide ECM fungi to the oak seedlings in our case. ECM fungal inoculation is frequently reported to increase the survival rate of ECM tree seedlings in places without ECM fungi, such as bare areas [19,51,52]. Furthermore, the invasion of black locust is a problem worldwide [53], and therefore, our findings would contribute to rehabilitation not only in China but also other countries, such as Europe.

5. Conclusions

Our study suggests that sowing seeds with oak forest soils can improve the water and nutrient conditions of seedlings by providing ECM fungal inoculum. Transplanting nursery seedlings also improved survival rate, but based on the ease of the method, we recommend sowing seeds to promote the succession of black locust to oak forests.

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