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Micropropagation of a West African wild grape (*Lannea microcarpa*)

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ABSTRACT

The propagation of *Lannea microcarpa* (an African wild grape) by seeds causes considerable variation in the offspring, making very difficult the selection, and multiplication of superior genotypes of interest. The species is also very difficult to propagate using conventional asexual technique. Therefore, micro cutting explants of young shoots from seedlings of *Lannea microcarpa* were grown *in vitro* on semi-solid Woody Plant Medium (WPM) supplemented for each experiment with three cytokinins at seven different concentrations for shoot formation and development. The cytokinins tested were 2iP (2-isopentenyladenine), TDZ (Thidiazuron), Zea (Zeatin) plus 0.05 µM IAA in the medium. Two subcultures were performed at 35 days interval after the initial *in vitro* culture establishment. Shoot development occurred primarily from axillaries buds formation that was greatest on a medium containing 56 µM of 2iP (plus 0.05µM IAA), with an average number of 4.5 ± 0.3 shoots per single node cutting and an average length of 4.7 ± 0.7 cm. After removal from culture, the shoots were induced rooting using IAA (Indole-3-Acetic Acid) and IBA (1*H*-indole-3-butanoic acid) at seven different concentrations. Shoots rooted in response to treatment with auxins (IBA and IAA) in the WPM medium and also in talc. The WPM medium produced an average number of 7 ± 0.5 roots per cutting at 32 µM IBA and with nearly 4.5 ± 0.7 cm in length. But the 1.5% of IBA in talc gave a better result with a higher number of 7.5 ± 0.4 roots per cutting with 5.5 ± 0.6 cm in length. The objective of this study is to develop a reproducible protocol for the rapid propagation of *Lannea microcarpa* in a semi-solid WPM medium supplemented with various concentrations of hormones.

Keywords: *In vitro* propagation, Sahelean tree, Cytokinins, auxins.

INTRODUCTION

Lannea microcarpa is one of the most important species in Africa south of Sahara. The species belongs to the family of *Anacardiaceae*. It is a Soudano-Sahelean species also present in the Guineo-Congolese region.

This species has a great socioeconomic importance in Burkina Faso. It is a source of food for both human (fruits) and livestock (forage), and used in local handicrafts (woodcarving, dyeing, and tanning). The bark contains tannin (Sereme et al., 1995a; 1995b). The plant is a real panacea: the parts (leaves,

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bark and fruits) are used in the composition of diverse formula in traditional pharmacopoeia (Kerharo and Adam, 1974). *Lannea microcarpa* ripe fruit epicarp which is dark red is assumed to be an interesting food colorant. This color is due to the presence of anthocyanins pigments.

The pressure on the use of the species is exacerbated by the high population of the Sudano-Sahelian region. This resulted in a depletion of once rich and dense stands degradation and sometimes their total disappearance. The failure to see the lack of recovery of the species, results in the continued deterioration which is aggravated by drought, wildfires, excessive cutting and its stray animals reported by several authors (Le Houerou, 1992; Unep, 1996; Hiernaux and Gerard, 1999). In addition, the species is overfished (skinned or limbed) for the purposes of traditional medicine and fodder for livestock. So the species is endangered. To address this problem, it must be restored by forestry (reforestation, conservation, maintenance, regeneration, etc.).

The seed of the species belongs to the group of seed called "recalcitrant". It is very difficult to maintain because of its high water content. It is also easily attacked by insects during storage. In addition, the species is characterized by great genetic variability. The propagation of the species by seeds causes considerable variation in the offspring, making difficult the selection, and multiplication of superior genotypes of interest. Vegetative propagation aims at reproducing offspring of genetically identical plants from a single plant source (Rout et al., 2006). This technology would be a good alternative method and a useful tool for mass-scale production of selected clones for genetic improvement of high quality varieties of the species, in a relatively short period. Previous experiments on horticultural cuttings of the species have not yielded satisfactory results (Loada, 2004; Sereme et al., 2008). That is why this species is particularly interesting to find another alternative for it propagation.

The purpose of the present study was to develop an efficient reproducible protocol for the rapid propagation of *Lannea microcarpa* by using micro-cutting of young shoots as explants in a semi-solid WPM medium supplemented with various concentrations of cytokinins and auxins.

MATERIALS AND METHODS

Plant material

The explants used in this study were taken on *Lannea microcarpa* Engl. and K. Krause which belongs to the *Anacardiaceae* family. These explants were micro-cutting with 3 cm of length and 0.5 - 0.7 cm diameter of young shoots with a single node. The shoots came from seedlings grown in a greenhouse in clean conditions.

Explants surface sterilization

The micro-cutting were immersed in 70% aqueous ethanol for 3 min and transferred to 50% sodium hypochlorite solution (Clorox 5.5%), supplemented with 2 to 3 drops of detergent or wetting "Tween 20" for mechanical agitation for 20 min. The cuttings were then washed 3 times with bidistilled water and blot dried on a sterilized filter paper prior to transferring in the medium in aseptic conditions under a laminar flow hood.

Culture establishment

The culture medium was a semi-solid agar containing mineral base and vitamins mix of WPM medium with sucrose 30g l^{-1}. The pH was adjusted to 5.8.

The medium is dissolved and homogenized at 121 °C for 10 min at 1.26 cm^{-2} and then, 20 ml dispatched into each 25 x 150 mm culture tubes. It is autoclaved again for 15 min at the same conditions for sterilization. All growth regulators addition is done, prior to autoclaving. Culture were grown in a growth room maintained at 24 ± 2 °C and were continuously exposed to a 14 – hr photo period at 80 - 100 µmol s^{-1} m^{-2} (400 – 700 nm) from cool-white fluorescent lights. Various concentrations of the cytokinins 2iP,

TDZ and Zea (0; 2; 10; 32; 56; 77; 99 µM) plus 0.05 µM IAA were used for bud induction and shoot multiplication. For shoot cuttings rooting, the auxins IAA and IBA were used at the following concentrations, 0; 2; 10; 32; 56; 77; 99 µM for the WPM media and, 0; 0.5; 1; 1.5; 2; 2.5; 3% for cuttings dipping in talc powder experiment. This experiment should allow selecting the best cytokinins and auxins and their concentrations in terms of highest shoot and root elongation and proliferation for *in vitro* micropropagation of *Lannea microcarpa*.

Shoot length and multiplication

After a period of 35 days from the initial culture establishment date, all the plantlets were taken out, one culture tube after another, and placed on a sterilized Petri-dish. The number of shoots and their respective lengths were measured and recorded. All of the procedures were done under strict aseptic techniques and conditions as the contaminations of any culture would require it to be discarded. After the measurements were completed, all the plantlets were cut in microcuttings and transferred to culture tubs containing new semi-solid WPM medium according to their respective cytokinins concentrations. From the first subculture, the steps were repeated after another 35 days.

Microcuttings rooting

For the rooting, microcuttings were removed from shoot multiplication culture and replaced in media containing various concentrations of auxins IBA and IAA (0; 2; 10; 32; 56; 77; 99 µM). In the experiment with talc, a thin sliver of the stem tissue is removed from the cut base prior dipping in the talc containing the following concentrations of IBA and IAA (0; 0.5; 1; 1.5; 2; 2.5 and 3%). Shoot microcuttings were then placed upright in vermiculite moistened with distilled water in a covered transparent plastic box and placed under the same growing conditions of temperature and light.

Acclimatization

After rooting, acclimatization of microcuttings is done in an enclosed greenhouse with decreasing moisture over time from 98% to 70% atmosphere. This means that the seedlings were maintained for 3 days in an atmosphere of 98% humidity and 7 days at 90%, 14 days at 80% and finally 70% for the remaining time until the seedlings become enough strong to be transferred in outside natural conditions.

Data analysis

The experiment was carried out in a completely randomized design with 10 replications and one cutting per tube for each treatment (hormones concentration). Data on length and number of roots shoots were recorded. The statistical analysis involves the analysis of variance and comparison of means by calculating the least significant difference. Separation of means after analysis of variance is made according to the Newman-Keuls test, $p < 0.05$.

RESULTS AND DISCUSSION

Shoot length

Lannea m. buds started to form within one week from the day of culture establishment. The success rate of shoots formation was an average of 98%.

In Figure 1, curves have similar patterns. From the seven concentrations used for the 3 cytokinins, the media containing a concentration of 56 µM (plus 0.05 µM IAA) was found to be the best for stimulating shoot length followed by 77; 99; 32; 10; 2; and 0 µM for the 3 cytokinins. The media containing a little amount of auxin are more effective than the auxin-free media. The highest shoot length (4.7 ± 0.7 cm) is obtained with the medium containing 2iP followed by Zea (4 ± 0.5 cm) and TDZ (3.7 ± 0,6 cm), all containing 0.05µM IAA. This shows the relative effectiveness of 2iP for shoot elongation of the species. Newman-Keuls test ($p < 0.05$) allowed to distinguish means shoot length significantly different from each other based on the cytokinin concentrations tested.

While the mean shoot length increased from the concentration of 2 µM to 56 µM (the highest), it is started to decrease when the cytokinin concentration was further increased. The phenomenon is actually similar to a research on micropropagation of different banana cultivars using scalps where the shoot length was increased with higher BAP (benzyl amino purine) level until 22.2 µM after which the shoot length also began to fall (Shirani et al., 2010). According to Chai et al. (2010), 2iP stimulate root growth, especially at lower concentrations, prior to the second subculture but the present study hadn't shown any induction and root growth during the second subculture with 2iP. A research on the regeneration of sugarcane using various cytokinins (2iP), Benzyl adenine (BA) kinetin, TDZ, and Zeatin, has reported that 2iP is the most effective cytokinin for shoot elongation (Chengalrayan and Gallo-Meagher, 2001). This confirms the results of the present research. TDZ is synthetic and highly active (Schmulling, 2004); some studies showed that TDZ was effective and more active than zeatin especially in the micropropagation of woody plants (Lu, 1993); but data of the present study hadn't shown any significant difference between these two cytokinins for both shoot elongation and proliferation. Bates et al. (1992) and Murthy et al. (1998) found that TDZ can reduce shoot elongation. This result is confirmed by those of the present study, but the shoot elongation began decreasing only when the medium contains more than 56 µM TDZ (Figure 1).

Shoot multiplication

Cytokinin is one of the plant hormone crucial for plant growth and development and is known to promote plant cells division. Various types of cytokinins can also stimulate lateral buds growth and thus causing multiple shoot formation by breaking shoot apical dominance (Trigiano and Gray, 2005).

The relationship between mean shoot number and the cytoknin used is shown in Figure 2. A similar histograms trend is observed in this figure like previously for shoot length. The highest number of buds is formed in the media containing 56 µM (+ 0.05 µM IAA). 2iP gave the greatest number of shoots (4.5 ± 0.4) followed by Zea (4 ± 0.5) and TDZ (3.7 ± 0.2). The later two are not significantly different in terms of shoot numbers. The lower shoot number at concentration of 0 and 2 µM could be explained by the fact that explants usually require a period of adaptation to *in vitro* environment. When IAA concentration was varied from 0.05 µM to 6.0 µM in the media containing 56 µM of cytokinin, there was no significant difference in the number of buds induced. Thereafter, for routine bud induction, basal medium containing 56 µM cytokinin plus 0.05 µM IAA was used.

Previous studies involving others plants noted that relatively high concentration of cytokinins should be present to observe high multiplication rates (Kenneth, 1986). This observation is also shown by Sarwar et al. (1997) for apple, for which when cytokinin concentration increased, shoot number increased but shoots length decreased. Lobna et al. (2008) also noted that the use of high cytokinin levels was one of the most effective methods to reduce shoot and leaf growth and promote the formation of meristematic clusters. Some studies also demonstrated that cytokinins alone may not be able to significantly induce multiple shoot formation in some species. But the addition of auxins in synergy with cytokinins promotes better shoot proliferation (Chengalrayan and Gallo-Meagher, 2001). Another suggestion would be to supply combination of cytokinins. In a study concerning *Bauhinia vahlii*, a leguminous plant, the combination of TDZ and kinetin recorded significant increase in shoot numbers lasting for as 4 subculture periods (Bhatt and Dhar, 2000). Data of the present study fit these models. Indeed, a positive correlation between the shoot number and the cytokinins concentrations up to certain values of the concentration (56 µM plus 0.05 µM IAA) and then stagnation and a light decreased shoot number for the 3 cytokinins used in this study on *Lannea m.*, was

observed. The combinations of each cytokinin and little amount of auxin (0.05 µM IAA), gave the best results for shoot elongation and proliferation of *Lannea microcarpa*. But the effects of combinations of cytokinins only couldn't be verified.

The Figures 1 and 2 allow to conclude that 2iP plus 0.05 µM IAA is best suited for bud induction, shoot elongation and proliferation for *Lannea microcarpa*,

Root length

After the multiplication, rooting will start within 7 days and completed in 28 days. No root growth was observed in the control tubes, which mean it is possible that *Lannea m.* has very low endogenous auxin, and thus requires high concentration to induce root growth *in vitro*.

The two auxins (IBA and IAA) demonstrated similar descending histograms in all the seven tested concentrations in the WPM medium and the talc. The overall pattern of total root length was seen to be descending from the optimum concentration 32 µM (WPM) or 1.5% (in the talc) to the lowest concentration (0 µM or 0%), and decreasing again from 32 µM to the highest concentration (99 µM in WPM or 3% in the talc) and beyond. This could be translated as increasing auxins concentration up to 32 µM (WPM) and 1.5% (talc) stimulates root length, but below and beyond these values, the two auxins have negative effects on root elongation of the species. This situation occurred because the two auxins were able to induce root differentiation but above 32 µM (WPM) and 1.5% (talc) they inhibit the process which is confirmed by experiment done by Oliveiraa et al. (2008) on other plant species. IBA gave a length of 4.5 ± 0.6 cm against 3 ± 0.5 cm for IAA in WPM medium. The best results were obtained with the auxins in talc powder, 5.5 ± 0.6 cm for IBA and 3.5 ± 0.5 cm for IAA.

The average root length with the talc is higher than the one of the WPM medium for both auxins. That may be explained by the fact that one of the characteristics of *in vitro* plantlets is the tendency to assume heterotrophic rather than autotrophic mode of nutrition. And since the nutrients are abundantly supply in the WPM medium, they don't need to develop too much and long root to absorb nutrient from that medium, in contrary to those in the vermiculite moistened with distilled water (Figures 3 and 4).

Root multiplication.

Cuttings of the species produced fewer than 8 roots per explants with both auxins. The highest number of roots with IBA are 7 ± 0.3 (with WPM medium) and 7.5 ± 2 (in talc) and with IAA, they are 5 ± 0.4 (with WPM) and 6 ± 0.1 (in talc). The means number of roots were significantly different between the 2 auxins for both WPM medium and talc powder. The media containing IBA gave the best results. And also the root multiplication rate is better ex vitro with talc than in-vitro with WPM for the species (Figures 3 and 4).

In conclusion, the effect of auxins IBA and IAA at various concentrations on the micropropagation of *Lannea m.* using young shoot discovered that both IBA and IAA at 32 µM (WPM) and 1.5% (talc) to be the best for root induction and elongation with a net advantage for IBA in talc powder.

Acclimatization

After rooting, acclimatization of microcuttings is done in an enclosed greenhouse with decreasing moisture over time from 98% to 70% atmosphere. This means that the seedlings were maintained for 3 days in an atmosphere of 98% humidity and 7 days at 90%, 14 days at 80% and finally 70% for the remaining time. We found that the batch of seedlings from micro-rooted cuttings *in vitro* has a higher mortality rate (15%) than those rooted *ex vitro* (7%) during the acclimatization. This could be explained by the fact that *in vitro* formed roots need a longer time to become normal and strong than those formed *ex vitro*.

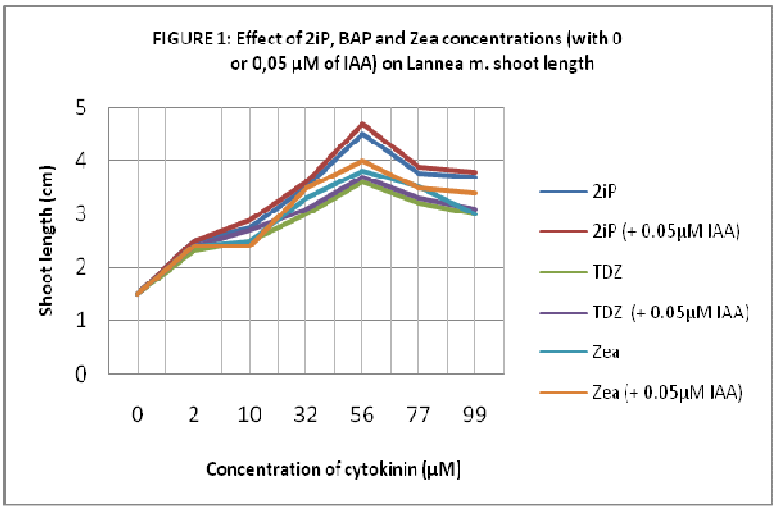


Figure 1: Average shoot length produced by *Lannea microcarpa* under different concentrations of 2iP, TDZ and Zea on semi-solid WPM medium (with 0 or 0.05% IAA) after 35 days of culture establishment.

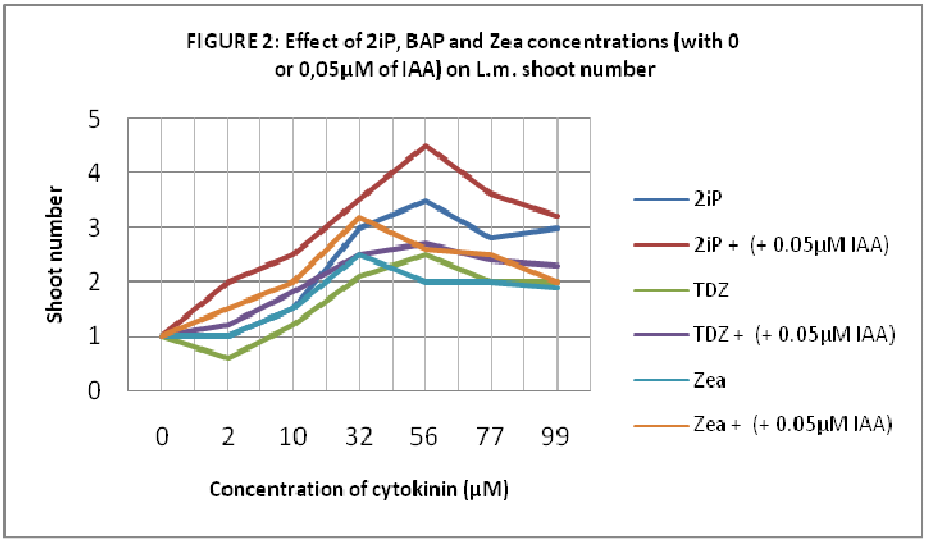


Figure 2: Average number of shoots of *Lannea microcarpa* under different concentration of 2iP, TDZ and Zea on semi-solid WPM medium (with 0 or 0.05% IAA) after 35 days of culture establishment.

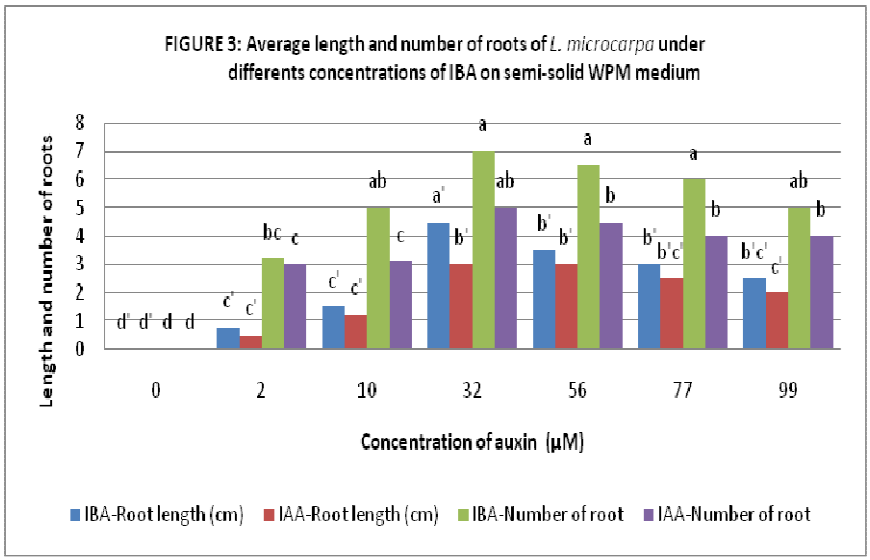


Figure 3: Average length and number of roots produced by *Lannea microcarpa* under different concentration of IBA and IAA on semi-solid WPM medium after 35 days of culture establishment. Histograms with a different alphabet have significantly different values according to Newman-Keuls test, $p < 0.05$.

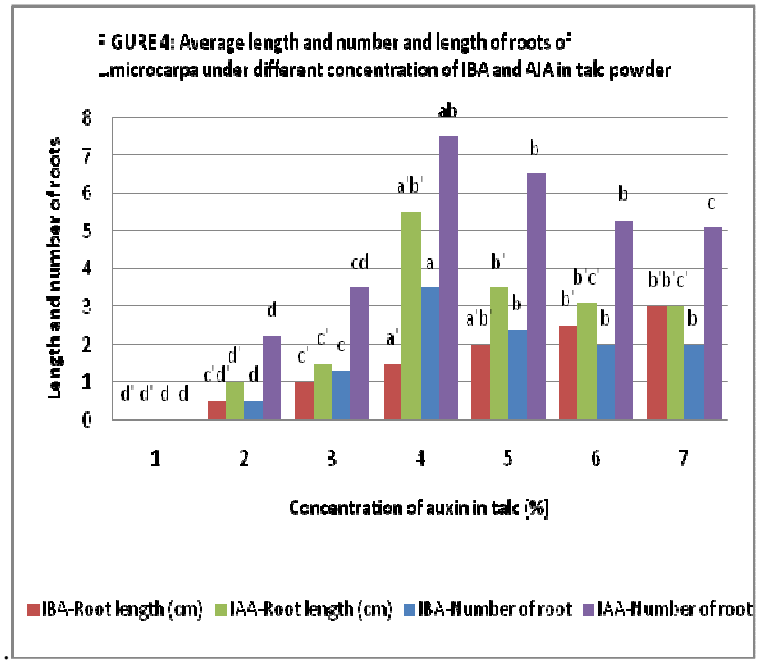
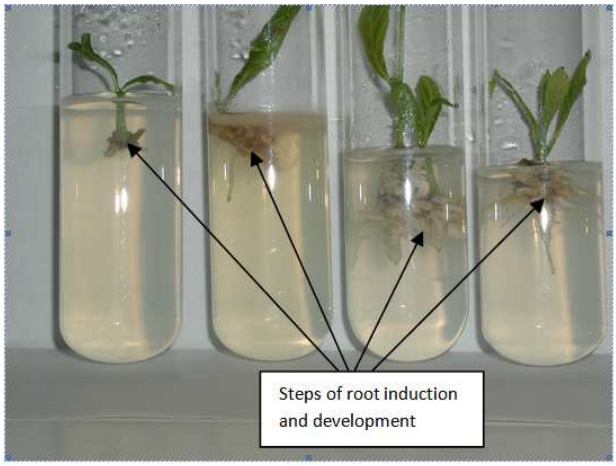


Figure 4: Average length and number of roots produced by *Lannea microcarpa* under different concentration of IBA and IAA in talc powder after 35 days of culture establishment. Histograms with a different alphabet have significantly different values according to Newman-Keuls test, $p < 0.05$.

Photograph 1: Culture establishment.

Photograph 2: Shoot proliferation.



Photograph 3: Microcuttings rootin.

Propagation protocol

The study allowed developing protocol for *in vitro* propagation of *Lannea microcarpa* as follow:

1) Place a single node cutting of the species in WPM medium with 56 µM of 2iP (+ 0.05 µM IAA), and subculture at least 2 times at 35 days interval for the multiplication of shoot. Each cutting included that a single node of the species will give an average number of 3 shoots. Therefore after harvesting, the micro cuttings explants could produce more shoots if transferred again to a new medium with the same composition.

2) After the multiplication, rooting will start within 7 days and completed in 28 days in WPM medium supplemented with 32 µM of IBA. But, for better results, it's recommended to use 1.5% IBA in talc.

3) The rooted plantlets will be then acclimatized in an enclosed greenhouse. This means that the seedlings should be maintained for 3 days in an atmosphere of 98% humidity and 7 days at 90%, 14 days at 80% and finally 70% for the remaining time until they become strong enough and the seedlings will be then transferred in the species real ecological conditions

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