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Initiation to the *in vitro* culture of *Pterocarpus erinaceus* Poir. in Togo

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Abstract
This study is focused on *P. erinaceus in vitro* culture and aims to improve the knowledge needed to contribute to its production. Plus trees seeds and softwood cuttings were used and the germination, resumption of development and growth of the aforementioned materials were monitored. The monitoring was conducted on Murashige & Skoog agar medium (MS0) under normal circumstances and on MS0 supplemented by growth regulators, auxin (MS-IBA) or cytokinine (MS-BAP). *In vitro* culture coupled with seeds going through a prior disinfection is successful with very low contamination (0-6%) and maximum germination capacity (90%). The seedlings growth monitored over 12weeks on MS0 is regular and characterized by a rejuvenation phenomenon observed in the leaves. There is also an aerial development after transplanting single-node segments from these seedlings on the same medium. Finally, for *in vitro* cutting, a positive reactivity of Plus trees cuttings cultured was illustrated by aerial morphogenesis. Based on the experimentations, MS0 is already proved effective. However, this result is accentuated through better budding percentages for 0.1mg/l IBA+0.5mg/l BAP medium. Furthermore, it is on 0.5mg/l IBA medium that the morphogenesis was more important. Finally, on all mediums, there were no rooting but instead a callus formation at the cuttings base.

Keywords: *Pterocarpus erinaceus* Poir, germination, growth, *in vitro* cutting, greenhouse softwood cuttings

Introduction
Global requirements for timber products are increasingly increasing as the area of productive natural forests is steadily shrinking [1] and seed multiplication of forest and agroforestry genetic resources for reforestation is the most widely used technique (simplicity and relatively low cost compared to vegetative propagation techniques). However, the use of seeds does not guarantee the maintenance of full genetic potential, especially when the goal is to multiply individuals with highly desirable traits. In West Africa and specifically in Togo, there is an emergency situation concerning *Pterocarpus erinaceus* Poir., a forest species with high economic and cultural value, linked not only to its multiplication but also to the conservation of its biodiversity [2] As one of the most exploited species in the countries of its range in West and Central Africa [3], there is a decrease in the plant genetic resources of the species accentuated by a slow regeneration (reduction in population density and endangered genetic heritage).
P. erinaceus propagation occurs generatively or sexually as well as vegetatively. However, although the seeds germination capacities obtained in the recent work of [4], and [5] have been found to be high, one of the major constraints born from the use of this mass production propagation technique, is in the slow rhythmic growth and relatively low shoot development of the seedlings after germination. For vegetative propagation, research results works of [6] and [7] brought forth stem cuttings without allowing the establishment of a specific route, reproducible and accessible. The scientific advances of Rabiou *et al.* [8] have shown that aerial layering is a solution for improving rooting and therefore for obtaining rooted plants, but large-scale production capacities of high-performance equipment are limited by the delicate installation of layering on the large trees of *P. erinaceus* [9], and the availability of high-quality sample trees due to wood-harvesting activities primarily targeting these phenotypically superior trees. Thus, since it is impossible to satisfy the ever-increasing demand on the local and international markets by using these traditional multiplication methods and at the same time preserve available genetic resources, there is an urgent need to optimize propagation and rooting techniques for the production of woody material and to set up reforestation areas, *ex-situ* plantations, and conservation strategies for *P. erinaceus* [10].
In developing countries, where there is a severe shortage of selected seed orchards, simplified *in vitro* cultivation techniques should be used to support reforestation programs. Indeed, the potential of biotechnologies remains underexploited in these countries, while their positive impacts have been demonstrated in developed countries in several sectors such as agriculture, agroforestry, and the agri-food industry [11], coffee [12]. Given the constraints associated with its natural spread, the efficient and economical multiplication of *P. erinaceus* in a short period of time could be met using *in vitro* culture [13, 14]; the first and most important step is the successful establishment of aseptic culture during the *in vitro* introduction of the species under consideration [15] and the assurance of the ability of the tree to regenerate.
Thus, in order to improve the available knowledge and contribute to the production of *P. erinaceus*, this study based on introduction to *in vitro* culture of this species was carried out. It focused on two aspects: expansion of variability by seed culture and conform multiplication through softwood-cuttings taken from trees with multi-performing criteria called Plus Trees (elite genotypes). This is the first step in the establishment of an efficient multiplication route through the *in vitro* culture of *P. erinaceus*.

Materials and methods

1. Plant material

The plant material for *in vitro* culture initiation has been harvested in the Togodo Reserve located in the Guinean zone in the south of Togo. It is a protected area of Ecological Zone V [16] which undergoes a subequatorial climate, with a long rainy season from March to July and a short rainy season from September to November. These two rainy seasons alternate with a long and a short dry season. The average annual rainfall is 1,000 to 1,200 mm and the average annual temperature is around 27°C [17, 18]. The harvested material consists of seeds extracted from fruit harvested from adult *P. erinaceus* trees, and softwood cuttings taken from greenhouse-grown Plus Trees stem cuttings.

2. In vitro culture from seeds

2.1. Seeds surface disinfection

The establishment of the most appropriate disinfection protocol for *in vitro* culture from *P. erinaceus* seeds was evaluated by performing three tests with disinfectant solutions with different concentrations and soaking time (Table 1). The seeds were surface-sterilized alternately in ethanol, yellow Bétadine® and Domestos (4.8 g sodium hypochlorite per 100g; Unilever® France; < 5% chlorinated bleaching agents, non-ionic surfactants, soap, perfume).

Table 1: Protocols used for disinfection of *P. erinaceus* seeds.

Solutions	Protocol 1	Protocol 2	Protocol 3
70° Ethanol	1 minute	1 minute	2 minutes
Yellow Bétadine® (Povidone iodine: 10g for 100ml)	100% Bétadine® 1 minute	80% Bétadine® 2 minutes	50% Bétadine® 3 minutes
Domestos (Sodium hypochlorite; 4,8 g for 100 g; Unilever® France)	10% Domestos 6 minutes	30% Domestos 4 minutes	50% Domestos 2 minutes
Sterile distilled water	6 rinses	6 rinses	6 rinses

2.2. In vitro germination

The disinfected seeds were sown in culture tubes (20 mm * 250 mm) closed with transparent plastic stoppers. Each tube contains 10 ml of MURASHIGE & SKOOG [19] (MS0) medium containing sucrose at 30 g/l, and solidified with agar (8 g/l); pH is adjusted to 5.70 ± 0.01. The germination was carried out with one seed per tube; the culture was placed in a growth chamber illuminated by white LED light tubes with a photoperiod of 16 hours of light with an intensity of 120 µE.m-².s^{-1}. The room temperature is adjusted to 25 ± 2°C. Three seeds replicates for each disinfection protocol were used.

In vitro germination is observed when the radicle emerges by rupturing of the seed coat and the counting has been carried out daily from the day of sowing for 21 days.

The following parameters were monitored:

- Cumulative contamination percentage (CCP),

$$CCP = \frac{Number\ of\ contaminated\ seeds}{Total\ number\ of\ seeds}\ x\ 100$$

- cumulative germination percentage (CGP),

$$CGP = \frac{Number\ of\ sprouted\ seeds}{Total\ number\ of\ seeds}\ x\ 100$$

- seeds germination speed expressed by the number of seeds germinated per day;
- and mean germination time (MGT)

$$MGT = \frac{N1J1 + N2J2 + \ldots + NnJn}{N1 + N2 + \ldots + Nn}$$

with N1 representing the cumulative number of germinations during the first day (J1), N2 the cumulative number of germinations during the second day (J2),…, Nn the cumulative number of germinations during the last day (Jn).

2.3. In vitro seedlings growth

The growth of sixty vitroplants from seed germination was monitored by the number of nodes, leaves, roots and the height of the vitroplants.

2.4. In vitro shoot regeneration from seedlings

After twelve weeks of growth, single-node cuttings with axillary buds dissected from *in vitro* seedlings were used as explants. Explants were grown individually in 10 ml of a culture medium contained in tubes (20 mm * 250 mm) closed with translucent stoppers. In order to assess the response to the *in vitro* culture, the experiment is carried out on a MURASHIGE & SKOOG medium (MS0) [19] free of plant growth regulators. A total of 72 explants are monitored over 4 weeks. The growing conditions remain unchanged in the culture room.

3. Plus Trees *in vitro* culture

In order to evaluate *in vitro* shoot organogenesis in *P. erinaceus*, stem cuttings taken on branches of *P. erinaceus* Plus Trees were grown in greenhouse. For these Plus Trees *in vitro* culture, softwood shoots dissected from greenhouse cuttings were used as explants and cultured in laboratory.

3.1. Greenhouse stem cuttings development and *in vitro* culture

In the Togodo protected area, stem segments were collected from the most accessible (lowest) Plus Trees branches. The samples were taken early in the morning, with a clean and sharp machete. The stem segments are defoliated and to prevent drying, then placed in moistened jute bags. The bags are placed in a cooler and then brought back to the greenhouse. In the greenhouse, these stem segments are cut into 30 cm long cuttings and disinfected by soaking them in 10% Domestos (sodium hypochlorite at 4.8 g per 100g; Unilever® France) solution for 2 minutes, followed by several rinses with tap water prior to cultivation in a substrate composed of a mixture of clean sea sand and JardiTropic® garden soil in 50/50 (v/v) proportions. Stem cuttings are monitored and regular watering is carried out to ease a resumption of aerial development. After budding and

development into leafy softwood shoots, the sample for *in vitro* culture was done considering the greenest and most vigorous shoots.

3.2. Plus Trees softwood cuttings disinfection

Softwood shoots collected from greenhouse cuttings were initially washed under a continuous stream of water for 15 minutes. They were then dissected into segments of 3 to 4 nodes for a complete immersion in disinfectant solutions. The segments are soaked in a 2% sodium hypochlorite solution for 30 minutes before moving onto disinfection under the laminar flow hood. Under the hood, the segments are first immersed for 1 minute in 70° ethanol with 2‰ tween 20, then 3 minutes in pure yellow Bétadine® (100%). They are then rinsed 4 times with sterile distilled water and soaked successively for 3 minutes in a 50% and 1 minute in 80% Domestos solution before ending with 8 rinses with sterile distilled water. After disinfection, the segments are dissected into single-node cuttings with one axillary bud and cultured on different mediums.

3.3. In vitro Plus Trees softwood cuttings growth

After disinfection, every single cutting is grown individually on 10 ml of culture medium contained in tubes (20 mm * 250 mm) closed with translucent stoppers with one explant per tube. To measure their development and assess their rooting abilities, cuttings are cultured on MURASHIGE & SKOOG (MS0) [19] agar medium supplemented with auxins (Indole-3-butyric acid or IBA) and cytokinins (benzylaminopurine or BAP): control MS0; IBA 0.5 mg/l; BAP 0.5 mg/l; IBA 0.1 mg/l + BAP 0.5 mg/l and IBA 0.1 mg/l + BAP 1 mg/l. Two repetitions of 13 cuttings or 26 cuttings are grown on each test medium. A total of 130 cuttings is monitored over 6 weeks.

4. Evaluated parameters and statistical analysis

The growth parameters measured are:

- The cumulative explant contamination percentage (CECP) corresponding to the ratio of the number of explants contaminated to the total number of explants put into the culture;
- The budding percentage (PD) corresponding to the ratio of the number of cuttings with active buds to the total number of cuttings put into the culture;
- The recovery percentage (PR) corresponding to the ratio of the number of cuttings with buds growing into leafy shoots to the total number of cuttings cultured.
- The size of the stem and neoformed shoots;
- The number of nodes and neoformed leaves;
- The number of neoformed roots.

Statistical analysis of the data was performed using R software version 3.1.2 [20]. An Analysis of Variance (ANOVA) was conducted to compare parameter averages and the Tukey Post Hoc Test, applied at the 5% threshold, was used to determine significant differences between the ANOVA group averages.

Results and Discussion

1. In vitro culture from seeds

1.1. In vitro germination

Amongst the three tested protocols, the best disinfection results with 100% of the seeds completely disinfected were obtained with Protocols 2 and 3. In contrast, for Protocol 1 seeds, contamination was recorded.

Considering the sanitization effect on the seeds germination capacity, Protocol 1 allows 90% germination to be recorded with an average germination time of 8.87 days with 6.67% of seeds contaminated. Protocols 2 and 3 eliminate 100% of microorganisms on the surface, resulting respectively in 80% germination in 9.28 days and 80% germination in 10.65 days. Protocols 1 and 2 seeds, therefore, germinate significantly ($p = 0.01$) faster in 9 days on average than those of Protocol 3 which take 24 hours more to reach the same percentage.

Considering the sanitization effect on the seeds germination capacity, it appears that although the contamination varies according to the protocols used, the germination capacities are high, 90% with no significant difference ($p = 0.59$). The effect of disinfectant solutions is recorded during the process where the seeds of protocols 1 (MGT = 8.87 days) and 2 (MGT = 9.28 days) germinate significantly ($p = 0.01$) faster, in 9 days on average, compared to Protocol 3 seeds, which takes an additional 24 hours (MGT = 10.65 days) to reach the same percentage.

For *in vitro* culture initiated from seeds, the three protocols can be used for *P. erinaceus* seeds because they have significantly reduced or even completely eliminated the pathogens responsible for contamination while maintaining a high germination capacity of 90% (Figure 1). The aseptic conditions created by the use of ethanol, Betadine and Domestos solutions simultaneously with the sterilised culture medium, therefore made it possible to render the disinfected seeds free of microorganisms whose presence and multiplication would considerably limit the normal growth of the species in culture [21, 22]. Indeed, the nature of the solutions used for surface disinfection varies according to the authors, but the most commonly used are sodium hypochlorite [23, 24] or calcium hypochlorite with a brief immersion in ethanol [25].

Analysis of the results also indicates that germination occurs in stages. For seeds disinfected using Protocol 1, the process begins 24 hours after seeding on the culture medium (Figure 1); the radicle starts to emerge through the seed coatings (radicle emergence). Twenty-four (24) hours later, on day 3, the first peak with a high germination speed is reached, and 13% is recorded as germination percentage. The first stage of germination is reached on day 7 with 36% of sprouted seeds and a germination rate that tends to cancel. It increases again from day 10, marking the start of new germinations with on average one seed germinated per day for 3 successive days. The speed reaches its second peak on day 12 and the next day, a landing is reached with 80% germination. 48 hours later, the last seeds germinate and the final mean germination percentage of 90% is obtained (Figure 1).

Seeds disinfected using protocols 2 and 3 start their germination 48 hours after sowing i.e. 24 hours after those from Protocol 1 (Figure 1). For Protocol 2 seeds, day 3 marks the beginning of germinations. On day 7, 30% germination is recorded and this proportion (30%) remains constant until day 9 when seeds are still germinating. The germination speed is the highest on day 11 when more than 2 seeds germinated simultaneously. A second stage is reached with 70% germination, then for the third stage a 76% germination is reached on day 14. Then, the final mean germination percentage recorded is 80% on day 19 as shown

by the last germination peak obtained. For the seeds disinfected with protocol 3, it is only on day 5 that we have the beginning of a rapid and continuous evolution of germination with on average one seed germinated until day 16. The proportion of 76.67% germination is recorded from that day to day 19, illustrating the first stage of the process. Finally, the last germinations are recorded on day 19, explaining the cancellation of the germination speed the next day. The average final germination percentage is 80% (Figure 1).

With Protocol 3 seeds, germinations are initiated on day 3 and the speed increases starting day 5. From day 5 to day 15 the rhythm remains relatively constant (on average one seed that germinates every day). This, results in the evolution of the germination percentage by a continuous curve without a plateau, unlike the observations for the seeds of the other two protocols. The plateau is recorded from day 16 to day 19, then the last seeds germinate on day 20, resulting in the recording of a germination capacity of 80%.

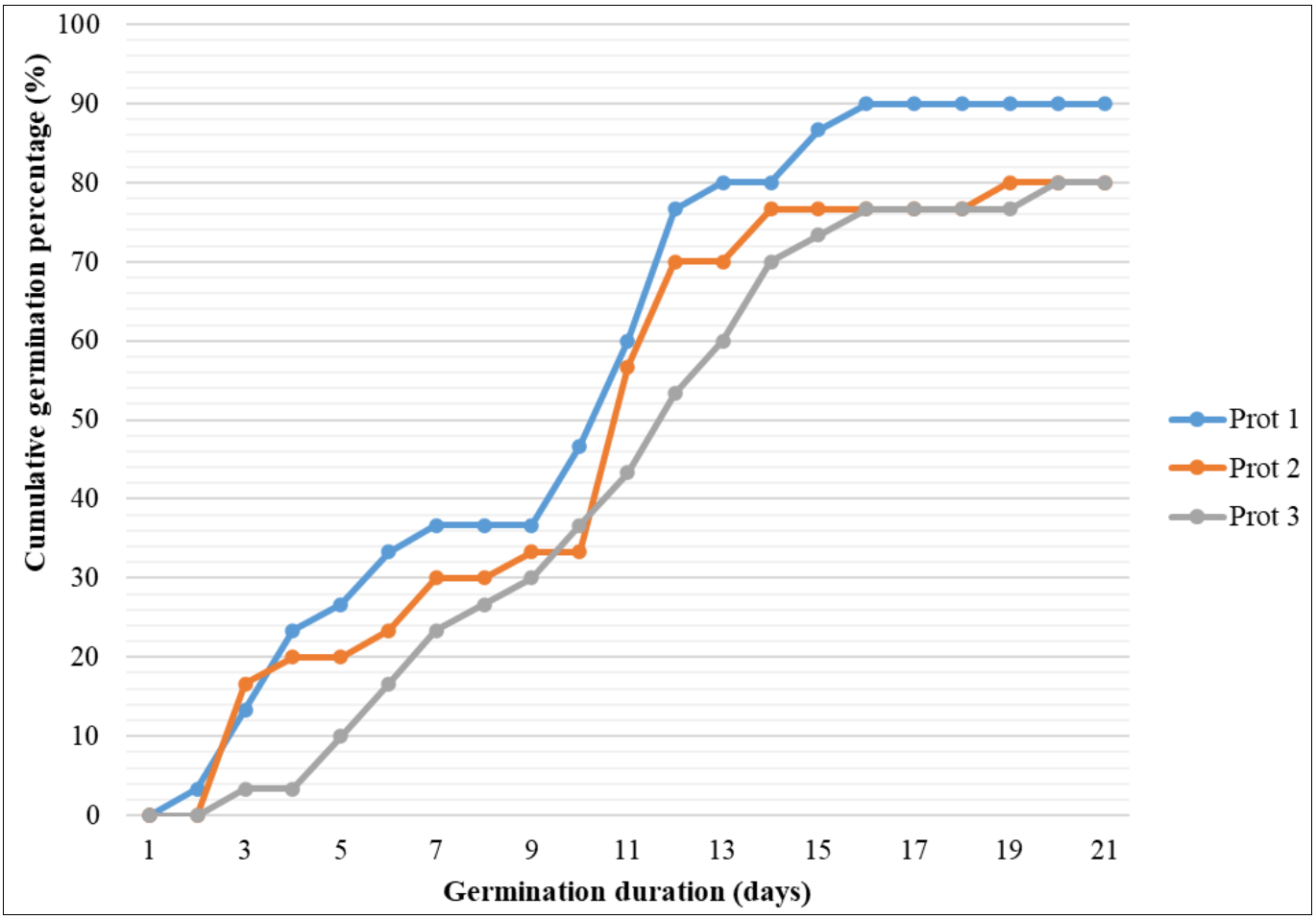


Fig 1: Evolution of the Cumulative germination percentage (CGP) for the *in vitro* germination of *P. erinaceus* according to disinfection protocols used

Seed disinfection treatments have had a beneficial effect on the microorganisms' elimination, as the percentages of contamination ranged from 0 to less than 10%. However, these treatments appear to have an effect on the development of the embryo, resulting in with a significant 24-hour extension of the mean germination time observed for seeds sanitized with Protocol 3 (MGT = 10 days) compared to that of seeds of Protocols 1 and 2 (MGT = 9 days). There is also staged germination characterized by the presence of several bearings, these are obviously observed during the recording periods of the maximum germination speed. Quashie *et al.* [14] obtained similar results by micropropagation of *Moringa oleifera* Lam. with *in vitro* introduction from seed. According to them, alternating periods of light and darkness on seeds germinated on the same culture medium, led to a high rate of germination in 2 steps.

Comparing the results obtained *in vitro* and greenhouse, two conclusions are reached (Table 2). First, seeds grown *in vitro* on agar mediums and in aseptic conditions, germinate significantly ($p = 0.022$) less rapidly than those grown in greenhouses on hydrophilic cotton. Indeed, the *in vitro* germination time varies from 9 to 10 days compared to what observed in the greenhouse (MGT = 5 days); the mean germination times are significantly longer when seeds are treated with disinfectant solutions and the values of this parameter (MGT) increase proportionally with the soaking time and the concentration of the solutions used. Then, disinfection prior to sowing on the MS0 agar medium does not affect the germinative capacity of the seeds (more of 80%).

Table 2: *In vitro* and greenhouse germination parameters of *P. erinaceus*

parameters	Greenhouse germination	*In vitro* germination
Mean germination time (days)	4.9 a	9.6 b
Cumulative germination percentage (%)	86.6 a	83.33 a

Tukey test: the values followed by the same letter in the line are not significantly different at the 5% threshold.

While increasing the soaking time may be detrimental to the survival of the embryo in the seed, as in *Cana indica* L. [26], it may also delay the time taken to complete the process, as in *Lawsonia inermis* L. [27]. Here, disinfection had an effect on the germination time of seeds sown on MS0 medium without affecting germination capacity.

2. *In vitro* seedlings growth

The growth of *P. erinaceus* vitroplants on the agar medium is characterised by a continuous and regular development over the 12 weeks of monitoring both at the aerial level (main stem, nodes and leaves) and at the subterranean level (development of the main taproot and the secondary roots or branches) (Figure 2).

Thus, for the number of nodes and leaves, sixty of the sprouted seeds i.e. more than 60% of the initial population, started the growth process already on the 21st day after sowing. The production of leaves and nodes of the monitored seedlings occurs from the first week and progresses throughout the experiment. In the analysis of the growth parameters, *P. erinaceus* being a species with alternate leaves, the number of leaves is, therefore, equal to that of nodes.

The leaf production and node kinetics are similar during growth monitoring (Figure 2A). In week 1, seedlings or vitroplants carry, on average, more than 3 nodes and 3 leaves. The following week, they develop one node and an additional leaf. The average rhythm of production of 1 leaf and 1 node per week remains constant. On week 12, seedlings have, on average 13.37 ± 5.87 nodes. This rhythm is also maintained with respect to leaf production. The loss of one leaf in week 7 resulted in the recording of 12.02 ± 5.16 leaves on week 12 (Figure 2A). In addition, the aerial development of the seedlings is characterized by the presence of leaves without leaflets (Figure 2B).

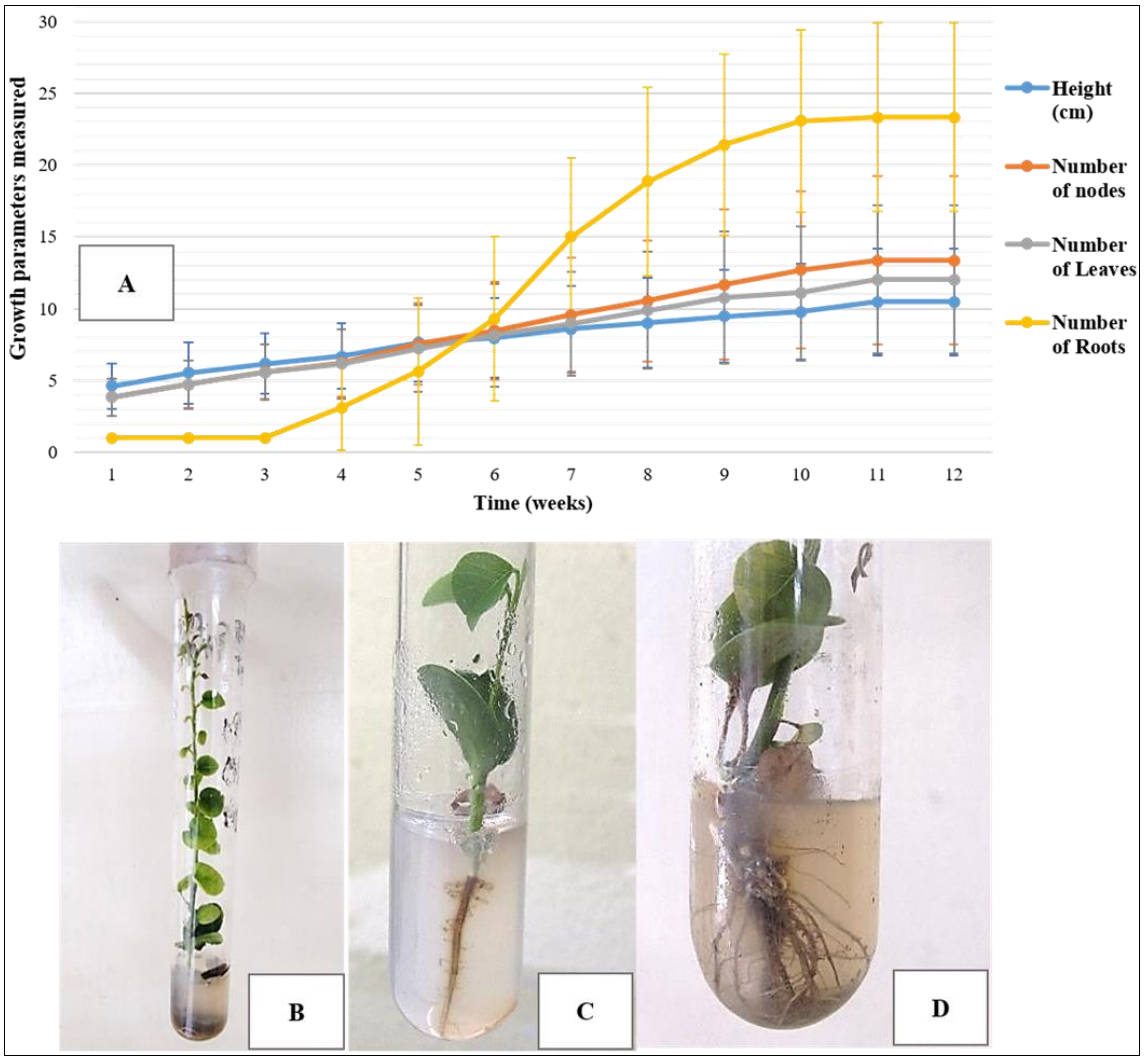


Fig 2: In *vitro* growth of *P. erinaceus* seedlings over 12 weeks on MS medium (*error bars = standard deviations*) A = Leaf and node development, Height growth and Root development; B = *P. erinaceus* seedling 12 weeks after sowing; C = Seedling with one main root and very young lateral roots 4 weeks after sowing; D = Seedling with numerous ramifications from the main root (longer adventitious roots) 8 weeks after sowing

The evolution of the seedlings stem height is characterized by a continuous and regular increase over the 12 weeks (Figure 2A). From week 1 to week 5, increasing from 4.61 ± 1.56 cm to 7.64 ± 2.74 cm, there was a total average gain of 3.03 ± 0.39 cm i.e. 0.8 cm (almost 1 cm) per week during this interval. From week 6 to week 12, the seedlings grow from 7.97 ± 2.74 cm to 10.47 ± 3.70 cm. The total average increase during the last four weeks is 5.5 cm corresponding to a weekly elongation of 0.42 cm, reflecting a slowdown of the initial speed.

For root development, the results show that after radicle emergence from the seed coat, it extends, becoming the main and pivotal root. This elongation lasts on average until the third week of growth (Figure 2C) and then secondary roots form, multiplying the total number of roots of the seedlings (Figure 2D). From week 4 onwards, the beginning of secondary root branching continues until week 11 on average (Figure 2A). The initial number of roots (one main root in week 1) is highly multiplied by week 7 (15.00 ± 5.51 roots) and continues to increase until it stabilizes in week 11. At the end of the 12 weeks of the experiment, seedlings have an average of 24 roots (Figure 2A).

From the original results of this study, it turns out that *P. erinaceus* adapts well to an introduction by seed and then to culture on agar medium. This habituation to trophic conditions, temperature, illumination and humidity, can be explained by the use of a nutrient medium (the MURASHIGE AND SKOOG medium) and external conditions thus creating an environment where the seedling finds all the elements necessary for its development; it can therefore easily express its morphogenetic abilities [19, 14]. Tiwari *et al*. [28] also observed harmonious development of seedlings from *Pterocarpus marsupium* Roxb. seedlings grown with light with a photoperiod of 8 h of illumination on simple MS medium. The only difference was in the light intensity of 55 $\mu mol.m^{-2}.s^{-1}$, which could explain why the growth parameters recorded after 40 days (a little over 5 weeks) appear to be lower (5 cm high with 4 nodes) than those measured for *P. erinaceus* (8 cm high with 8 nodes). Moreover, in this adaptation process, Quashie-Mensah Attoh and Ahama [29] mentioned the fact that the traits of plants grown *in vitro* will be essentially juvenile because of the property of plant cell totipotence, the basis of *in vitro* culture. This is consistent with the phenomenon of rejuvenation observed during the formation of leaves without leaflets, contrary to observations in natural environments [30, 31]. This rejuvenation also marks the successful *in vitro* introduction of *P. erinaceus*.

3. *In vitro* shoot regeneration from seedlings

Cuttings on MURASHIGE & SKOOG medium (MS0) showed a good reactivity after introduction onto the culture medium. Cuttings, which consist in restoring a new individual from a fragment of a plant on a "mother" subject that takes root, is one of the most common techniques [32] and when done in vitro the results are interesting [33, 34]. Thus, after 6 days, the first buddings were noticed and then the development into leafy shoots. After 4 weeks of culture, the maximum percentage of buds developed corresponding to the recovery percentage was 70.83% with 51 explants reactive on the initial 72 cuttings.

The mean value of shoots elongation was 1.75 ± 0.88 cm. Explants formed on average 3 nodes (3.30 ± 0.24) and 3 leaves (3.16 ± 0.29) without leaflets. None of the explants produced roots during monitoring. Translucent callus have been formed on the cuttings base in the agar medium. Instead, there were translucent calluses that formed on the basal part immersed in the agar medium.

P. erinaceus is a woody species with relatively slow *in vitro* growth, which is the case for woody species introduced *in vitro* [35]. Their *in vitro* regeneration or *in vitro* propagation is all the more difficult because of a rhizogenesis that is difficult to perform and which requires different regeneration strategies [36, 37]. Despite this difficulty of explants to produce roots, it is interesting to note their reactivity, activity resumption of the cuttings buds, and aerial development, in a medium without growth regulators. For *Pterocarpus marsupium* Roxb., the best developmental recoveries were observed on MS medium compared to those obtained on B5 [38] and White [38] media in the Tiwari *et al*. [28] trials, in accordance with the preliminary work of Kalimuthu and Lakshmanan [40] and Rath [41].

The results obtained in this study prove the capacity for the development of *P. erinaceus in vitro* and the prospects for the development of an environment adapted to the growth of cuttings from seedlings obtained after sowing [42, 43].

4. Plus Trees *in vitro* culture

4.1. *In vitro* budding of Plus Trees softwood cuttings

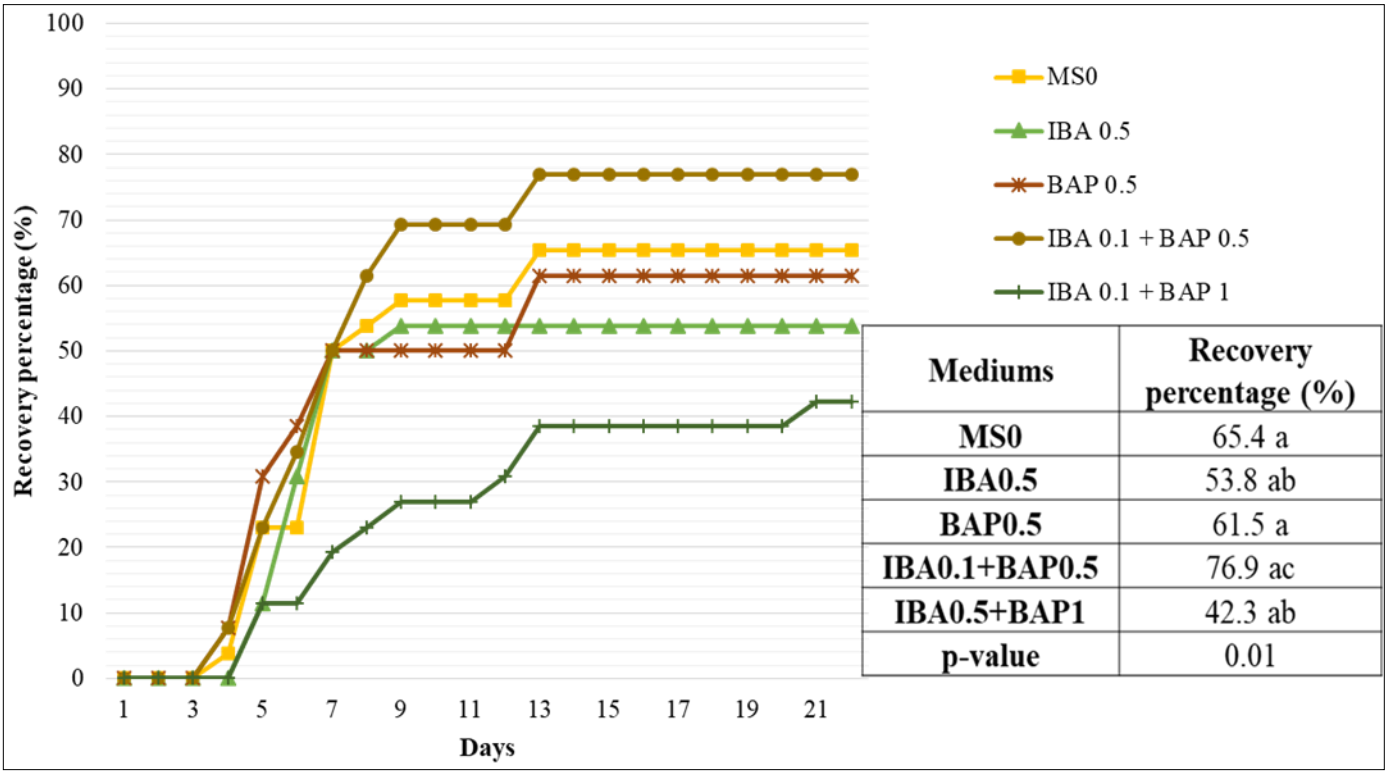
The use of disinfectant solutions proved to be essential for the propagation from cuttings of Plus Trees coming from the outdoor environment (greenhouse). This disinfection reduced the proportion of pathogens on explants in the greenhouse, which varied significantly ($p = 0.01$) depending on the culture media used. The contamination percentages recorded are on average 43.88% on culture mediums. Specifically, more than half of the cuttings cultured on the 0.5 mg / l BAP-supplemented medium have been contaminated, while the lowest significant ($p = 0.01$) contaminations have been observed on the IBA-supplemented medium (0.1 mg/l) + BAP (0.5 mg/l). On the other mediums, the contamination percentage recorded has been 46.2% (Table 5). Considering provenance, with explants from different locations (greenhouse or outdoor), Dorion *et al.* [44] obtained better disinfection with samples taken from mother plants or node cuttings material grown in the greenhouse. The recorded contamination percentages varied from 24% to 42% with the use of 70° alcohol, calcium hypochlorite (50-100 g/l) and tween 80 (100 ppm).

The cuttings in culture showed reactivity to the conditions tested, illustrated by a development in the axillary node of the segments. The response period varies from 6 to 9 days with an average of 7.4 days; the statistical tests performed do not reveal any significant difference depending on the culture medium.

The cuttings development resumption was carried out in three phases in particular a latent phase where nothing is visibly happening. The resumption and development evolution curves have the sigmoidal allure, characteristic of the kinetics of growth composed of three phases. Theses tree phases are latency stage (where there is no visible manifestation), acceleration stage (where visible changes are observed as a result of the specific mechanisms implementation, depending of the organ) and ending of the process [45, 46]. Then the massive buds development is recorded, and finally, there is the end of the process (Figure 3). Thus, from day 1 to day 3, no resumption of axillary bud activity is detected for any cuttings. Recovery begins on day

4 for cuttings on MS0 medium and those on 0.5 mg/l BAP and 0.1 mg/l IBA + 0.5 mg/l BAP mediums. On 0.5 mg/l IBA and 0.1 mg/l IBA + 1 mg/l BAP mediums, bud development is initiated starting day 5. During the second phase, the cuttings bud actively and quickly lasts on average until day 9. The bud development percentage is high, increasing, on average from 7 to more than 50%. The percentages increase very quickly in 5 days from 3 to 57% for cuttings on MS medium, from 7 to 50% for cuttings on 0,5 mg/l BAP medium, from 7 to 69% for those on 0.1 mg/l IBA + 0.5 mg/l BAP medium. For the 0.5 mg/l IBA and 0.1 mg/l IBA + 1 mg/l BAP mediums the percentages were respectively from 11% on day 5 to 53% and 26% on day 9 (Figure 3).

Finally, from day 13 until day 22, the plateau phase occurs. Within this timeframe, bud development slows down and come to an end. In this timeframe almost 10% of cuttings on MS0 medium (control) still develop and the final percentage recorded is 65.4%. For cuttings on 0.5 mg/l IBA medium, the maximum percentage obtained is 53% and for those on 0.5 mg/l BAP medium, it is 61.5%. Budding percentages recorded are 76.9% for 0.1 mg/l IBA + 0.5 mg/l BAP medium and 42.3% for 0.1 mg/l IBA + 1 mg/l BAP medium (Figure 3).



Mediums	Recovery percentage (%)
MS0	65.4 a
IBA0.5	53.8 ab
BAP0.5	61.5 a
IBA0.1+BAP0.5	76.9 ac
IBA0.5+BAP1	42.3 ab
p-value	0.01

Fig 3: Evolution of the cuttings recovery percentage according to culture medium. *MS0 = MURASHIGE & SKOOG medium; IBA = Indole-3-butyric acid (auxin); BAP = 6-benzylaminopurine (cytokinin)* Tukey test: In the table, the values followed by the same letter in the column are not significantly different at the 5% threshold.

The axillary buds budding was greater with for MS-AIB+BAP medium than for the medium MS0. The recovery percentage has been relatively important (more than 40%).

4.2. *In vitro* Plus Trees softwood cuttings growth

Considering the aerial development of cuttings, the results obtained indicate on MS0 medium and growth regulators (auxin IBA and cytokinins BAP) supplemented mediums, that cuttings grown into caulogene and callogenic plants (Figure 5). No shoots were obtained from any of the buds. The shoots proportion formed from buds development was the lowest on the 0.5 mg/l BAP medium; only 3.8% of explants saw their bud develop to one shoot on the 100% cultured ($p = 0.002$). It increased to 11.5% with 0.1 mg/l IBA + 1mg/l BAP medium ($p = 0.03$). Shoots formation was much more important on the 0.5 mg/l IBA-alone medium (30.8%), and the proportion decreased on 0.1 mg/l AIB + 0.5 mg/l BAP (23%), and on the MS0 mediums (15%) (Table 3).

After 6 weeks, the cuttings carried, on average one shoot except on 0.5 mg/l BAP medium (medium exclusively supplemented with cytokinin), where two shoots were recorded (Table 3). The length of the shoot is on average 2 cm and the addition of growth regulators has no significant effect ($p = 0.31$) on this parameter. Length of the shoot varies from 2.7 cm on the medium MS0 to 1.6 cm for at 0.5 mg/l IBA medium. The number of nodes is maximum on the MS0 medium (4 nodes on average) and weaker on other mediums i.e. 2 nodes produced. One of the most surprising results is that there is fewer (1-2 leaves) or even no leaf production on new shoots of cuttings. Rare leaves were observed on cuttings in culture on MS0 medium (Table 3).

Table 3: Growth parameters and aerial development characteristics of *P. erinaceus* cuttings according to culture mediums.

Mediums (mg/l)	Recovery percentage (%)	Leafy shoots percentages (%)	Number of shoot	Shoot height (cm)	Nodes	Leaves
MS0	65.4 a	15.4 b	1.0 a	2.7 a	4.0 a	1.2 a
IBA 0.5	53.8 ab	30.8 a	1.0 a	1.6 a	2.7 ab	0.1 a
BAP 0.5	65.1 a	3.8 c	2.0 b	2.0 a	2.0 ab	0 a

IBA 0.1 + BAP 0.5	76.9 ac	23.1 ab	1.0 a	1.7 a	1.8 b	0 a
IBA 0.1 + BAP 1	42.3 ab	11.5 b	1.0 a	1.3 a	1.7 b	0 a
p-value	0.01	0.002	$2.2.10^{-16}$	0.311	0.043	0.161

MS0 = MURASHIGE & SKOOG medium; IBA = Indole-3-butyric acid (auxin); BAP = 6-benzylaminopurine (cytokinin)
Tukey test: the values followed by the same letter in the column are not significantly different at the 5% threshold

Finally, when considering the parameters characterising morphogenesis, recovery percentage, number and height of shoots, number of nodes and leaves, it appears that the MS0 medium is already performant by similar values (recovery and characteristics of shoots) or even superior (formation of nodes and leaves) compared to media enriched with growth regulators (MS-IBA, MS-BAP and MS-IBA+BAP). However, it is interesting to note a generalized aerial morphogenesis on all mediums tested. The hormone-free medium (MS0) was more favourable to bud development, although the percentage was relatively low (18%). The endogenous and exogenous hormonal balance between growth regulators, particularly auxins and cytokinins, plays a major role in the control of the explants organogenesis in culture [34]. In addition, the reactivity of the starting plant material also depends on several factors linked essentially to the physiological state of the explants, but also to the nutritional and environmental conditions of the crop [47].

It is however interesting to note a generalized caulogenesis on all the tested mediums. The 0.5 mg/l IBA medium was the most suited for the formation of shoots from axillary buds while the 0.5 mg/l BAP medium recorded the highest shoots production (2 shoots). The combination of the two growth regulators seems to inhibit shoots development and this result would increase proportionately with the BAP concentration. Thus, just as the presence of an endogenous hormonal balance led to a considerable reduction in the number of shoots and nodes of uninodal segments of *Pterocarpus marsupium* grown on MS medium enriched with BAP and ANA (naphthalo-acetic acid) in the *in vitro* propagation work of Tiwari *et al.* [28], so too was the inhibition of bud development in a medium characterized by a hormonal combination was recorded for axillary buds of *Aristolochia longa* L. [48] with 0% for budding percentage on MS medium supplemented with 0.5 mg/l IBA + 1 mg/l BAP after 6 months of culture.

The reactivity of the cuttings was manifested first by the development of the lateral buds and their growth into shoot, followed by cellular dedifferentiation marked by a calluses formation. From the 15th day after cultivation these calluses began to proliferate on cuttings. Cuttings showing calluses over the entire length of the segment were found to constitute 46-65% of all the cuttings. The least proportion of calluses i.e. 46.2%, was recorded on 0.1 mg/l IBA + 1 mg/l BAP medium. In contrast, the highest percentage of callogenesis i.e. 65.4% was recorded on 0.1 mg/l IBA + 0.5 mg/l BAP medium. On the other three mediums, it was respectively 46.2% for 0.5 mg/l IBA, 50% for 0.5 mg/l BAP and 53.8% for MS0. Two types of callus have been formed (Figure 4):

- Friable cream white calluses (FCW calluses) observed at the apex of the cutting at the section area made by the scalpel and also on the aerial part of the cutting;
- Whitish to greenish compact calluses (GCC calluses) located on the area immersed in the agar medium.

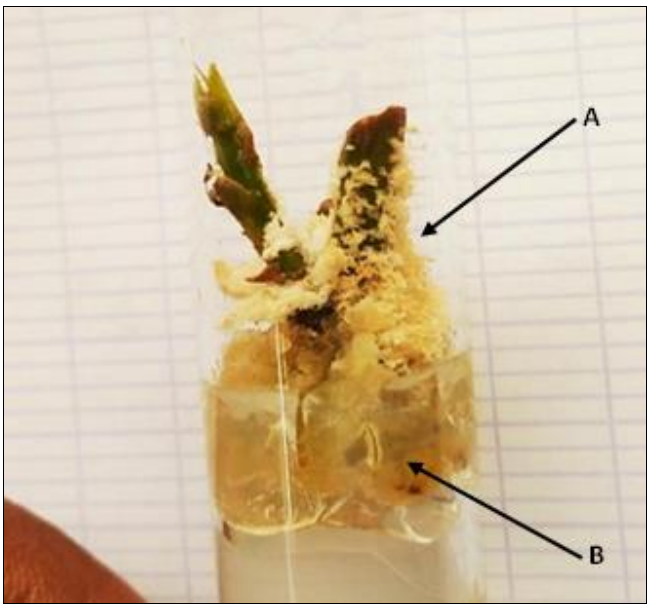


Fig 4: Type and characteristics of calluses developed on *in vitro* cuttings
A = Friable cream-white calluses observed at the apex of the explant (FCW); B = Compact calluses located on the submerged part (GCC).

The majority of the cuttings with calluses (30 to 75%) showed the association of these two types of calluses which would be scarred for the friable and root for the compact one. Friable calluses-alone cuttings were not recorded (Table 4) compared to cuttings wearing only compact callus.

Table 4: Distribution of calluses types formed on cuttings of *P. erinaceus*.

Mediums	FCW cuttings	GCC cuttings	Both calluses cuttings
MS0	0%	69.2%	30.8%
IBA 0.5	0%	25%	75%
BAP 0.5	0%	38.4%	61.5%
IBA 0.1 + BAP 0.5	0%	47.1%	52.9%
IBA 0.1 + BAP 1	0%	45.5%	54.5%

MS = MURASHIGE & SKOOG medium; IBA = Indole-3-butyric acid (auxin); BAP = 6-benzylaminopurine (cytokinin)
FCW callus = Friable cream white callus; GCC callus = white to greenish compact callus

In the scientific studies for improving in vitro micropropagation of *Pistacia vera* L., transplanting nodal segments from woody branches of adult trees every fortnight onto a medium containing BAP (1 $mg.l^{-1}$) significantly reduced infections, and the development of callus at the explant section area was also observed [47]. Nanson [49] results indicate that the calluses frequently occur at the microcuttings base in the early stages of *in vitro*

culture. This back to the juvenile characteristics of the specialized cells (dedifferentiation and massive and rapid divisions) of the stem, would allow a reorientation towards a root organization process; the hardest thing is to find the optimum medium and the hormonal stimulations adapted for this purpose [50]. These results are an essential reactivity determination of *P. erinaceus in vitro* culture, predicting an adaptation to biotechnology technologies.

Conclusion

The introduction to *P. erinaceus in vitro* culture was carried out with seeds and softwood stems from individuals located in the protected area of Togodo in southern Togo in the Guinean zone. This introduction was made to assess the response to morphogenesis and *in vitro* multiplication capabilities for large-scale propagation of elite individuals and the conservation of the plant genetic resources (biodiversity preservation). *In vitro* introduction is an essential step for the application of the various techniques of plant biotechnology that remain to be explored.

Firstly, the study of aseptic culture establishment conditions with *P. erinaceus* seeds allows to conclude that the protocols tested were effective without weakening germination capacity which has remained high (90%). The seedlings monitored over 12 weeks grown relatively slowly. An interesting regularity was noted in the development of the aerial and root organs coupled with a rejuvenation phenomenon observed in the leaves. For the *in vitro* shoot regeneration from seedlings, single-node stem cuttings isolated from *P. erinaceus* seedlings and cultured on MURASHIGE & SKOOG medium showed a positive reactivity, with an aerial development. Four weeks after germination, there was morphogenesis i.e. the formation of new shoots with nodes and leaves. Secondly, assessment of direct organogenesis capacities using *in vitro* cutting with softwood cuttings taken from the greenhouse and necessarily disinfected before culture on agar media, gives a positive result. The MS0 medium is efficient and softwood cuttings showed aerial morphogenesis, and the best budding percentages were recorded for cuttings cultured on 0.1 mg/l of IBA (auxin) + 0.5 mg/l of BAP (cytokinin) medium. Following the budding, the most important aerial development was measured on 0.5 mg/l AIB medium; for the root organogenesis, a callus formation at the cuttings base base without development into a root system. In the end, the results obtained in this study are a contribution to *P. erinaceus in vitro* culture using germination or direct organogenesis by *in vitro* cutting techniques. They allowed to the determination of a disinfection methodology by the additional use of Bétadine® yellow and Domestos and the morphological development description for the two types of explants used.

Further studies are needed for *in vitro* cutting and shoot regeneration techniques, which is based on the species' intrinsic regeneration capacities, in order to overcome the difficulties of initiating of rhizogenesis from explants. This is an essential step in establishing a complete, effective and reproducible procedure for high quality plants of *P. erinaceus*.

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