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Critical factors affecting an efficient micropropagation protocol for *Pyrus spinosa* Forskk.

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Abstract

Almond-leaved tree is one of the most commonly found native forest species in Greece, exhibiting valuable properties and thus suitable for multipurpose silviculture. Several critical factors were studied for the development of a successful micropropagation protocol of *Pyrus spinosa* juvenile trees. Newly immerged shoots of three-year-old plants, after their surface sterilization, were established on a modified MS nutrient medium (thiamine-HCl 1 mg L^{-1}, nicotinic acid 1 mg L^{-1}, pyridoxine-HCl 1 mg L^{-1}) with 5 μM BA. Clean explants were transferred in the multiplication stage on a novel medium (Pear Medium 1), by adding 10 μM BA especially developed for *Pyrus* species. Due to poor culture development, the effect of photosynthetic photon flux density (PPFD) on the improvement of regeneration was studied. The exposure of explants to 10 μmol m^{-2} s^{-1} for the first two weeks followed by exposure to 35 μmol m^{-2} s^{-1} for another two weeks, was proved essential for the good development of cultures promoting both multiplication and elongation of explants. For further enhancement of shoot regeneration, the use of Pear Medium 1 with five different combinations of growth regulators was tested (BA, IBA). The most beneficial for the development of good quality shoots was 5 μM BA+0.0246 μM IBA (4.67±0.40 new shoots per explant, elongation 1.28±0.13 cm). As multiplication was mainly based on axillary branching and the production of new shoots was still relatively low, the orientation of explants (horizontal vs upright position) in relation to the medium was investigated. Regeneration of shoots almost tripled, reaching 13.67 new shoots per explant in the case of horizontal orientation after the removal of the apical part (0.2 cm). The most successful rooting procedure (rooting: 83.33±5.89 %, root no: 6.20±0.49 roots per plantlet, root length: 0.56±0.05 cm) consisted of an initial stage of root induction maintaining microshoots in complete darkness for seven days. The rooting medium was a modified MS (½ NH_4NO_3, ½ KNO_3) supplemented with 24.6 μM IBA. Microshoots were subsequently transferred to a root development stage in the same rooting medium without auxin, exposed to 10 μmol m^{-2} s^{-1} for another four weeks. Successful acclimatization (87.5 %) was achieved after six weeks on perlite.

Key words: *Pyrus spinosa*, almond-leaved pear, *in vitro* regeneration and rooting, photosynthetic photon flux density, acclimatization.

Introduction

Pyrus spinosa Forskk. is a native forest species of Greece, member of the *Rosaceae* family. It is characterized by the almond shaped form of its foliage reflecting its older scientific name *P. amygdaliformis* Vill. Almond-leaved pear is one of the most commonly found wild forest tree of the greek countryside, grown at low and medium altitudes, at road sides, paths, at the edges of cultivated fields and pastures. It is particularly a xerophile species, well known for its adaptability in various climatic and soil conditions even at their most extremities (Zohary, 1997; Procopiou and Wallace, 2000; Falcinelli and Moraldi, 2001).

Currently, *P. spinosa* is attracting scientific interest as many valuable properties condensed in this single forest species, rendering it ideal for multipurpose silviculture. Among its many properties, the use of its fruit in various edible forms (fresh, dried and for other culinary uses) is the most known of all. Pharmaceutical and cosmetic industries are widely using antioxidant substances found in almond-leaved pear (Tzanakis *et al*., 2006; Hu *et al*., 2009; Kundaković *et al*., 2014). *P. spinosa* is also used as rootstock for grafting of edible pear varieties (Procopiou and Wallace, 2000), as well as an ornamental and honey plant (Falcinelli and Moraldi, 2001). Moreover, it is included in the list of world economic plants (Wiersema and Leon, 2016). As a result, there is an immediate need for the development of mass propagation techniques of which the most reliable is micropropagation for selected genotypes of *P. spinosa*.

In general, most *Pyrus* species exhibit recalcitrance in *in vitro* manipulations. Several researchers have studied and focused on factors, such as special nutrient requirements for genus *Pyrus,* especially in the shoot multiplication stage (Grigoriadou *et al*., 2000; Kadota and Niimi, 2003; Thakur and Dalal, 2008; Reed *et al*., 2013b, c; Wada *et al*., 2013). Reed *et al*. (2013a), after an extensive study on nutrients for five different genotypes of three *Pyrus* species, designed and proposed three types of a novel nutrient medium namely Pear Medium that proved beneficial for all.

Although there is a plethora of research on the regeneration through tissue culture of edible varieties and rootstocks of many *Pyrus* species to date (Lane, 1979; Yeo and Reed, 1995; Kadota and Niimi, 2003; Thakur and Dalal, 2008; Thakur and Kanwar, 2008a, b; Reed *et al*., 2013a, b, c), for *P. amygdaliformis=P. spinosa* the available bibliography is limited (Dolcet-Sanjuan *et al*., 1990a, b; Dolcet-Sanjuan *et al*., 1992). The micropropagation protocol proposed by Dolcet-Sanjuan *et al*. (1990a) is the only one found for this particular species, only a part of their research work conducted among other three *Pyrus* genotypes and one of *Cydonia oblonga* L.

Given the fact that there is no recent publication on tissue culture for the species, the present work focuses on the investigation of particularities and critical factors affecting *in vitro* regeneration of young *P. spinosa* trees. Consequently, a simple and efficient micropropagation protocol is proposed for the rapid production of vegetative material of the species.

Materials and methods

The plant material of *P. spinosa* was young native trees, 3 years of age, originated from seeds, growing in pots provided by the greenhouse of the Forest Botanic Garden, Aristotle University of Thessaloniki, Greece. The plants were maintained under controlled environmental conditions (16 h photoperiod, temperature 24±1°C and light intensity 35 μmol m^{-2} s^{-1}) in a plant growth chamber. After ten days, the newly emerged shoots, 2-3 cm long, were isolated and used as explants. Surface sterilization was conducted as follows: firstly the explants were rinsed with running tap water for 1 h 30 min with the addition of Tween 80 followed by their soaking in a mixture of antioxidants (300 mg L^{-1} ascorbic acid and 200 mg L^{-1} citric acid) for 16 min and a 7 min soaking in 12 % v/v NaOCl. Then, shoots were rinsed three times for 5 min with sterilized-distilled water and established *in vitro* on a modified MS medium (Murashige and Skoog, 1962) (thiamine-HCl 1 mg L^{-1}, nicotinic acid 1 mg L^{-1}, pyridoxine-HCl mg L^{-1}) (Dolcet-Sanjuan *et al*., 1990a) supplemented with 5 μM BA and 7.1 g L^{-1} agar (Sigma) in glass culture tubes (15 cm, Ø 2.5 cm). Medium pH was adjusted to 5.7. Cultures were maintained for 4 weeks in a plant growth chamber with fully controlled environmental conditions, as described above.

Multiplication stage: Clean explants were transferred for multiplication on Pear Medium 1 supplemented with 10 μM BA in baby jar food vessels (Sigma) (height 9.85 cm, Ø 5 cm) in the above culture conditions. Subculturing was conducted every 4 weeks on the same medium until an adequate number of explants was available for the experimental work. Pear Medium 1 was used in all the experimental work of multiplication and rooting stages, unless it is otherwise stated.

Effect of photosynthetic photon flux density (PPFD) on multiplication and growth of explants: PPFD was investigated in order to obtain good quality culture growth. Three variants of PPFD were tested: a) low, 10 μmol m^{-2} s^{-1} for the first 2 weeks followed by explant exposure to 35 μmol m^{-2} s^{-1} for another two weeks, b) medium, 35 μmol m^{-2} s^{-1} for four weeks and c) high, 100 μmol m^{-2} s^{-1} for the same period. PPFD was recorded by using a Sekonic L-398 photometer.

Effect of growth regulators on shoot multiplication: Different concentrations of BA (5, 10 μM) with or without 0.0246 μM IBA were tested for shoot multiplication and elongation.

Effect of explant orientation on nutrient medium for the improvement of shoot regeneration: In order to further increase shoot multiplication, explants 3-4 cm in length, were placed on the medium with 5 μM BA as follows: a) upright position of explants (control) b) and c) horizontal position after the removal of 1 or 0.2 cm of the apical part, respectively.

Rooting experiment: Newly immerged microshoots, 3-4 cm, were used for rooting experiments. The procedure included two substages, depicted in Table 1, namely: a) an initial stage of root induction in complete darkness and b) a stage of root development on a rooting medium without auxin along with the exposure of cultures in 10 μmol m^{-2} s^{-1} for the first 2 weeks and in 35 μmol m^{-2} s^{-1} for another 2 weeks, unless it is otherwise stated.

Table 1. The rooting procedure of *P. spinosa.* Different treatments tested in the rooting stage

Treatment	Initial stage of root induction	Stage of root development
R1 (Control)	-	Pear Medium 1
R2	Pear Medium 1 + 32 μM BA. 7 days in darkness	Pear Medium 1
R3	Pear Medium 1 + 32 μM BA. 24 h in darkness	a) ½ Pear Medium 1 b) ½ Pear Medium 1+ 3 g L^{-1} activated charcoal
R4	Modified MS for rooting (½ NH_4NO_3, ½ KNO_3)+24.6 μM IBA 3 days in darkness	Transfer to Magenta GA-7 vessels (Sigma) with perlite. Modified MS for rooting +2.5 g L^{-1} Phytagel™ (Sigma)
R5 a)	Modified MS for rooting without auxin. 7 days in darkness	Modified MS for rooting. PPFD 10 μmol m^{-2} s^{-1} for 4 weeks
b)	Modified MS for rooting + 24.6 μM IBA. 7 days in darkness	

Acclimatization stage: Rooted plantlets were transferred for hardening in a small greenhouse of plexiglass (46.5 cm x 26.5 cm x 30 cm). Plantlets were established on sterilized perlite in plastic nursery trays under the controlled environmental conditions of the plant growth chamber. Each plantlet was covered with plastic transparent vessel constantly for the first three weeks to maintain high relative humidity followed by a three-week gradual hardening to environmental conditions. Acclimatization of plantlets was conducted under low PPFD 10 μmol m^{-2} s^{-1}. Every 20 days a modified liquid solution of MS macronutrients (½ NH_4NO_3 and ½ KNO_3), was applied.

Statistical analysis. Thirty explants in all multiplication experiments and 20 for *in vitro* rooting were used per treatment. The experiments were repeated twice. Experimental data were subjected to analysis of variance (ANOVA) by using SPSS V22. Data in percentages were subjected to arcsin transformation prior to statistical analysis. Mean comparisons were made by using Duncan's multiple range test at $P \leq 0.05$.

Results and discussion

Clean explants were cultured on various standard nutrient media in the multiplication stage, such as MS, DKW and WPM in preliminary experiments with very poor regeneration of new shoots (data not shown). Based on the recent bibliography on Pear Medium, one of its forms (Pear Medium 1) was chosen to be applied for the first time on *P. spinosa*. The cultures responded positively resulting in the production of adequate plant material for experiments. Regeneration of young shoots was mainly based on axillary branching. Even though callus was formed at the base of the cultures, this callus was not productive.

Effect of photosynthetic photon flux density on multiplication and growth of explants: Due to developmental problems, PPFD was investigated in the multiplication stage and the results are shown in Fig.1.

Among the three different treatments tested, the most beneficial

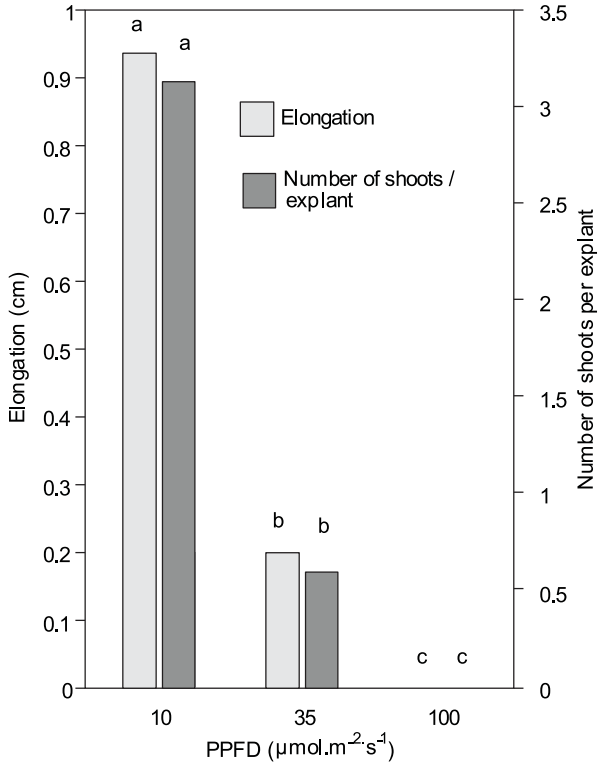

Fig. 1. Effect of PPFD on elongation and multiplication of explants. Mean values with the same letter are not significantly different according Duncan's multiple range test at $P< 0.05$.

treatment for the regeneration and growth of vibrant cultures was, maintaining in low light levels during the first two weeks followed by medium levels for another two weeks (Fig.1 and Fig. 2a, b). This is probably due to the low light requirements of the intact plant, as its foliage emerges very early in spring. Other researchers have also reported habits and properties of the whole plant retained and expressed in culture (Aranda-Peres *et al*., 2009; Pedroso *et al*., 2010).

Similarly, for three different *Pyrus* species, Yeo and Reed (1995) observed increased *in vitro* production of new shoots per explant under relatively low light levels (25 µmol $m^{-2} s^{-1}$). *Prunus avium* L. also exhibited low light requirements (25 µmol $m^{-2} s^{-1}$) for *in vitro* development of two mature clones (Scaltsoyiannes *et al*., 2009).

However, the results of the present work were in complete contrast with those of Dolcet-Sanjuan *et al*. (1990a) who, for the same species, reported significant increase of shoot multiplication *in vitro* under very high light intensity conditions (135 µE $m^{-2} s^{-1}$). In fact, when the cultures were exposed to PPFD of 100 µmol $m^{-2} s^{-1}$ all became brown and necrosed within a week, whereas at the medium level of 35 µmol $m^{-2} s^{-1}$ the cultures exhibited poor development and some necrosis. This is probably due to the different plant material of *P. spinosa* in study. In general, Hartmann *et al.* (2002) stated that under *in vitro* conditions, cultures respond differently as far as light intensity is concerned.

Low PPFD for the first two weeks was applied in all the experimental procedures of this stage.

Effect of growth regulators on shoot multiplication: The effect of growth regulators on the multiplication of almond-leaved pear was highly promoted by the combination of 10 µM BA with 0.0246 µM IBA (Table 2).

Table 2. Effect of growth regulators on elongation and multiplication of *P. spinosa*

Treatment	Elongation (cm) ±SE	Number of new shoots/explant±SE
0 µM BA	0.756±0.061 d	0.366±0.091e
5 µM BA	1.220±0.116 bc	2.133±0.258 d
10 µM BA	0.936±0.117 cd	3.133±0.384 c
5 µM BA+ 0.0246 µM IBA	1.280±0.133 b	4.666±0.406 b
10 µM BA+ 0.0246 µM IBA	1.620±0.132 a	7.766±0.634 a

Mean number ±SE. Sample number 30. Values of mean numbers followed by the same letter are not statistically significant for $P \leq 0.05$ (test Duncan).

However, at this concentration of cytokinin, various morphological disorders were observed on our cultures such as: hyperhydricity, fasciated shoots, tissue hypertrophy and shoot tip necrosis. High BA concentration, one of the responsible factors for such problems, is a great obstacle to the normal micropropagation procedure for *Pyrus* species (Lane *et al*., 1998; Kadota and Niimi, 2003; Poudyal *et al*., 2008; Liu, 2009; Aygun and Dumanoglu, 2015) resulting in loss of cultures and economic viability of the process. Based on this fact, the combination of 5 µM BA with 0.0246 µM IBA was proposed as the most suitable (Fig. 2c) for multiplication. This is in accordance with Thakur and Kanwar (2008a, b) for *P. pyrifolia* grown *in vitro* that despite observing the best new shoot regeneration with the addition of 2 mg L^{-1} BA in medium, finally proposed a lower concentration of BA (1.5 mg L^{-1} BA) due to similar developmental problems of their cultures. For the same reasons, growth regulator concentrations analogous to those of the present study are also proposed by other workers for the micropropagation of various *Pyrus* species such as: *P. calleryana* Dcn (Berardi *et al*., 1993), *P. syrica* (Shibli *et al*., 1997) and *P. communis* var. *pyraster* (Caboni *et al*., 1999).

For *P. amygdaliformis,* however contrary to the findings of the present work, Dolcet-Sanjuan *et al*. (1990a) reported increased production of new shoots per explant by respective increase of cytokinin (BA) concentration from 5 µM to 10 µM and 20 µM in combination with high light intensity. In their study, however, researchers did not comment on any morphological malformations on these high cytokinin concentrations.

Due to the fact that some morphological disorders still persisted even in the proposed cytokinin concentration, the cultures were no longer sealed with parafilm. This practice resulted in the full recovery of cultures to normal development, as it facilitated natural aeration. An analogous procedure for the alleviation of hyperhydricity problems existed in cultures of four different *Pyrus* species was proposed by Poudyal *et al*. (2008). The researchers, apart from the reduction of cytokinin levels, used cotton at the mouth of the flasks and thus completely eliminated malformations.

Effect of explant orientation on nutrient medium for the improvement of shoot regeneration: Another limiting factor for the successful *in vitro* propagation for many *Pyrus* species is the regeneration trend that mainly depends on axillary branching resulting in their limited ability for shoot production per explant (4-6 new shoots per explant) (Thakur and Kanwar, 2008a; Haq and Kaloo, 2010; Ružić *et al*., 2011). The same applies for our cultures with comparable new shoot production. This is attributed to apical dominance maintained on the explants probably due to increased levels of endogenous auxin levels pre-existing in the

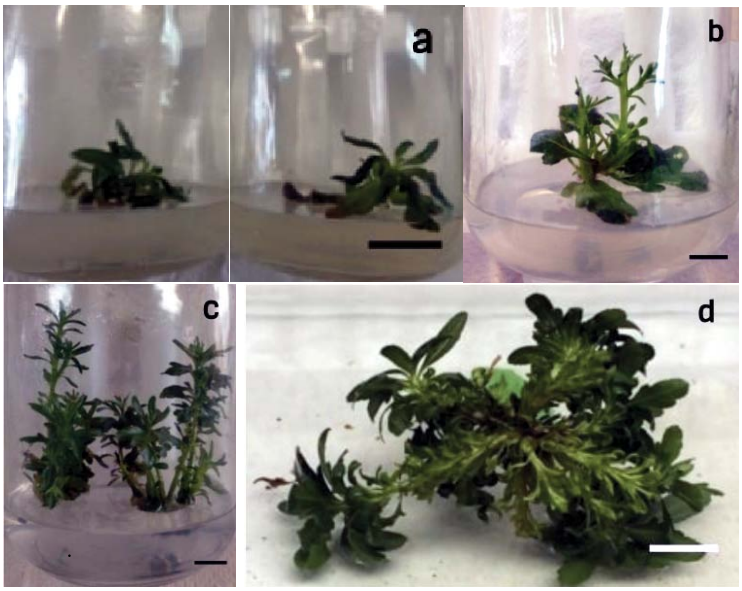

Fig. 2. Shoot regeneration of *P. spinosa* after four weeks on Pear Medium 1 with 10 µM BA. a) under 35 µmol m^{-2} s^{-1}; b) under low PPFD (10 µmol m^{-2} s^{-1} for the first two weeks); c) induction of axillary shoots, *in vitro*, grown on Pear Medium 1 with 5 µM BA and 0.0246 µM IBA; d) close view of newly emerged axillary shoots from a decapitated explant placed horizontally on the nutrient medium. Scale bar 1 cm.

tissues (Shen and Mullins, 1984). In order to further improve shoot multiplication, the orientation of explants (horizontal or upright) on the medium in combination with their decapitation was investigated.

According to the results, the production of new shoots per explant was almost tripled (13.67±0.24a) when the original decapitated shoot (0.2 cm) was placed horizontally on the medium compared to the control (4.66±0.40c). In the case of horizontally placed shoot with 1 cm of the apical part isolated (8.40±0.40b), production was also increased but clearly less than in the previous treatment (Fig. 2d).

The above results were in accordance with those of Lane (1979), who initially proposed this practice and reported significant increase in the production of axillary shoots of *P. communis* L. Bartlett *in vitro*. Dolcet-Sanjuan *et al*. (1990a) noticed only a slight increase in shoot multiplication by placing 2 cm explants horizontally on the nutrient medium coupled with high BA concentration and high light intensity.

Rooting experiment: According to the results in Table 3, the treatment R5b performed the best rooting percentage with good developed rooting system (root number and elongation) (Fig. 3a) by following the procedure of Dolcet-Sanjuan *et al*. (1990a) for almond-leaved pear.

The above researchers reported for *P. amygdaliformis* 90 % rooting and the development of a well-formed rooting system by adding 32 µM IBA. They also observed abundant callogenesis at the basis

Table 3. Effect of different treatments on *in vitro* rooting of *P. spinosa* Forssk

Treatment	Root (%)	Root number	Root length (cm)	Callogenesis	Comments
R1	18.75±6.17d	2.65±0.076d	1±0.067b	none	-
R2	41.2±7.78b	8.85±1.04a	0.83±0.051c	high	-
R3a	0±0e	-	-	high	-
b	0±0e	-	-	none	Necrosis
R4	20.0±6.32d	4.5±0.08c	1.20±0.018a	low	Necrosis
R5a	33.3±7.45c	5±0.16bc	0.65±0.072d	none	-
b	83.33±5.89a	6.2±0.49b	0.56±0.055d	high	-

Mean number ±SE. Sample number 20. Values of mean numbers followed by the same letter are not statistically significant for $P \leq 0.05$ (test Duncan).

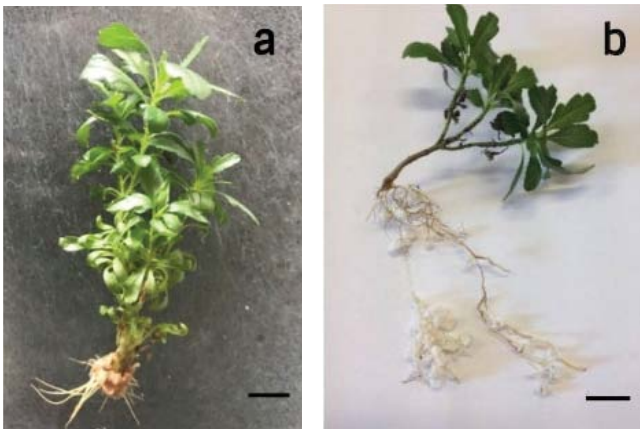

Fig. 3. *P. spinosa* rooted plantlets. a) five weeks after *in vitro* rooting manipulations; b) six weeks after acclimatization. Scale bar 1 cm.

of plantlets, as in the present study. Other researches also reported successful *in vitro* rooting results by following similar procedures for various *Pyrus* species: Haq and Kaloo (2010) for *P. pyrifolia* (Burm. f.) Nakai., Hassanen and Gabr (2012) for *P. betulaefolia* and Saadat *et al*. (2012) for *P. glabra* Boiss.

Acclimatization stage: In the acclimatization stage, high plantlet survival (87.5 %) was recorded after their six-week hardening on perlite (Fig. 3b) by following the procedure proposed by Shibli *et al*. (1997) for rooted plantlets of *P. syrica*. The above researchers reported 95 % survival in the acclimatization stage by covering plantlets with glass jars or plastic sheets compared to open pots (70 %). Successful acclimatization of rooted plantlets was reported for various *Pyrus* species such as: 50 % (Shen and Mullins, 1984), 73 % (Bhojwani *et al*., 1984) and 83-90 % (Al-Maarri *et al*., 1994). Dolcet-Sanjuan *et al*. (1990a), for the same species, do not give any information on acclimatization. Transferring of hardened plantlets in soil is in progress. Currently, this protocol is applied on mature trees of *P. spinosa* with desirable characteristics in order to investigate novel uses of the native biodiversity.

This work is a new approach for the rapid multiplication through plant tissue culture techniques of *P. spinosa*, a recalcitrant, multipurpose forest species with very limited research available so far.

Key factors, essential for the synthesis of a simple and functional micropropagation protocol revealed that: the use for the first time of Pear Medium 1 supplemented with 5 µM BA and 0.0246 µM IBA successfully applied for the *in vitro* multiplication of almond-leaved pear. Best shoot regeneration achieved by horizontal orientation of the explants along with their slight decapitation. The most successful rooting procedure was accomplished on MS with half-strength NH_4NO_3 and KNO_3 by adding 24.6 µM IBA and keeping microshoots in darkness for a seven-day period. Then, microshoots were transferred in an auxin free rooting medium for another four weeks. Finally, plantlets were efficiently acclimatized in a six-week period maintained in the growth plant chamber on perlite. Low light levels were required for the first two weeks during multiplication and through the whole period of *in vitro* rooting development and acclimatization stages.

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