https://doi.org/10.48047/AFJBS.6.13.2024.6553-6561

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

In vitro Propagation For the Conservation of *Ilex aquifolium* Threatened by the Proliferation of Axillary Shoots

Khater Nadia[1.2*], Benahmed Amira[3], Benbellkacem Ibtissem[3], Charouana khaoula[3], Benchikhelhocine Imen[3], Daas Abla[3]

[1]Departement of Ecolgy and Environment, Faculty of life and Sciences, Université of Batna 2 Algeria

[2] Biodiversity, Biotechnology, and Sustainable Development Research Laboratory "BBDD" Algeria

[3] Research Center of Biotechnology, Constantine, Algeria

***Corresponding author**: n.khater@univ-batna2.dz

Volume 6, Issue 13, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.13.2024.6553-6561

Abstract

Ilex aquifolium L. is an endemic species in Algeria's Tellian and Saharan Atlas mountains in the country's center and east, and it is on the verge of extinction owing to a variety of reasons. To help preserve it, we devised an *in vitro* micropropagation technique. The cuttings with axillary buds were cultured on different media supplemented with different combinations and concentrations of plant growth regulators. The best results were obtained on Woody Plants Medium (WPM) supplemented with 6-benzylaminopurine (BAP: 1.5 mg/LL, 3 mg/L) and naphthalene-acetic acid (NAA: 1.5 mg/LL) in combination with BAP (1.5 mg/ L); BAP (1.5 mg/ L) with Gibberellins (GA3: 0.5 mg/ L). The best shoot proliferation rates were obtained on WPM supplemented with 1.5 mg/LL (BAP) and BAP: 1.5 mg/L; GA3: 0.5 mg/L; or BAP: 1.5 mg/L + ANA: 1.5 mg/L. The highest rooting rate (100%) was obtained with shoots grown on WPM medium supplemented with NAA (1.5 mg/LL). Absence of routing formation was observed in media containing indolacetic acid (IAA: 1 mg/ L) alone or combined with BAP: 0.5 mg/ L) The best-rooted shoots must be acclimatized to a substrate consisting of a mixture of peat and sand (1:1 v/vv), the seedlings have been acclimatized successfully.

Key words: Ilex aquifolium L., axillary buds, *in vitro*, micropropagation, WPM, growth regulators.

Introduction

Ilex is a member of the Aquifoliaceae family. Tropical and temperate regions of the world are home to more than 500 identified species. Most of them are shrubs or trees, either deciduous or evergreen (Hu, 1989). English holly, as it is generally called, has long been used as an ornament due to its attractive leaves and distinctive crimson fruits. Many of its hybrids and cultivars are well-known in Europe as landscape plants and for their cut branches used as holiday decorations (Galle, 1997).

Ilex aquifolium is native and widespread in Europe, North Africa (Algeria, Moroco, and Tunis), West Asia, and China; it is also commonly grown in Northwestern (United States) (Bonner and Karrfalt, 2008), and in Australia, Victoria (British Columbia) (Harrington and Reichard, 2007).

In Algeria it is a naturally occurring species that grows slowly and is endangered. It is crucial to protect it in order to encourage regeneration in the positive areas (Yahi et al., 2008).

Due to its involvement in a variety of forest communities and its relationship with geopedological, climatic, and anthropological characteristics, as well as its ability to produce vigorous discharges of foliar masses that make it useful as a species of hail, it is regarded as a habitat of community interest and plays a significant role in biological and ecological diversity (Brunu, 2011).

According to Hartmann et al. (2011), the genus Ilex can reproduce through seeds, cuttings, grafting, air layering, and micropropagation, and the majority of research to date is focused on vegetative techniques for the large-scale propagation of superior phenotypes.

Throughout the year, a lot of plantlets can be produced quickly using the *in vitro* culture of forest trees (Shahzad et al., 2017). Two crucial processes *in vitro* propagation are root formation and shoot induction. The remarkable capacity for regeneration of plant somatic cells is highlighted by the fact that shoot growth is a crucial factor determining shape (Ikeuchi et al., 2016).

Nodal segments and leaves have been used in papers by Dang et al. (2011), Sun et al. (2010), Luna et al. (2003), Sansberro et al. (1999), Mattis et al. (1995), and Mahjada et al. (2000) to grow Ilex species vegetatively *in vitro*.

The study's objectives are to micropropagate whole *Ilex aquifolium* L. plants *in vitro* by starting organogenesis and to assess the effects of different growth regulators and hormone combinations on the induction of the explants of *Ilex aquifolium* in order to identify the ideal conditions for improved organogenesis and rooting. Preserving the species is another goal of the study.

1. Materiel and methods

1.1. Cultural initiation

Cultures were produced using seedlings obtained through embryo germination under aseptic conditions. Corps of *Ilex aquifolium* were collected in November 2018 in their natural habitat in the national parc of Belezma, Batna, East of Algeria (35°54'09" N and 6°25'62" E.1479 m.a.s.l.). Seeds were obtained by scaring of grapes; they were washed by running water tap for 20 min, and then rinsed with distilled water. The seeds were surface disinfected with ethanol (75% for 5 min) and then immersed in sodium hypochlorite (10 °C) for 15 min, and washed in sterile water three times (5 min for each).

1.2.Culture Induction

Zygotique embryos were excised under a steromicroscope in a laminar flow hood. These isolated embryos were cultured in culture pots that contained 70 ml of WPM (Lloyd and McCown, 1981) and MS (Murashing en skoog, 1962) media, supplemented with 3% sucrose and 0.6% agar. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 20 min. After these four embryos were placed per culture pot containing 70 ML of the WPM and MS media. Four embryos per pot were used for each medium, and all media were replicated ten times. Cultures were incubated at (25 °C) in the dark.

Three of the eighty embryos were germinated in WPM media after 10 weeks. For proliferation and elogantion, the germinated embryos were transferred onto WPM medium supplemented with 1.5 mg/L of AIB in order to promote further elongation and proliferation.

1.3. Treatments for micro propagation

Three-month-old seedlings were carefully stripped of one or two axillary buds from microcuttings measuring 1 or 1.5 cm in order to prepare them for micropropagation. Afterwards, the microcuttings were placed onto WPM supplemented with 1.5 mg/L of IBA, 1.5 mg/L + 0.5 GA3, and 3 mg/L of IBA.

1.4. Rooting of plantlets and acclimatization

The *in vitro* plants were placed in four different rooting media for the rooting phase: ½ WPM supplemented with 1.5 mg/L of NAA, 1 mg/L IBA + 1 mg/L IAA, and 1.5 mg/L IBA. During the first week of cultivation, cultures were started in complete darkness. In the last stage of cultivation, cultures were cultivated at 22°C with a 16-hour photoperiod. The *in vitro* plants were carefully taken from the pods, cleaned, and then transplanted into plastic pots containing peat and sand (1:1) after four to eight weeks of incubation in these mediums.

1.5. Data analysis

Data from shoot micropropagation and rooting studies were analyzed using the one-way analysis of variance (ANOVA), a multiple comparison based on a completely random design. P-values <0.05 were considered statistically significant. Tukey test was applied to determine exactly which treatments were different (p 0.05). All statistical studies were performed using SPSS Version 20-32bit.

2. Results

2.1. Induction phase

Micropropagation *in vitro* depends on the composition and concentration of base salts, growth regulators, and organic components (Ge et al., 2006). The reactivity of the starting plant material depends on several factors related mainly to the physiological state of the explants, but also to the nutritional and environmental conditions of the crop.

The effect of the growth regulators and culture media on the proliferation of *Ilex aquifolium* buds and leaves is summarized in Table 1.

Table-1. Effect of WPM medium and growth regulators on seedling mean length, number of leaves, and shoots

Medium	Growth regulators (mg/L)	Shoot length (cm)	Nbr. of leaves/plants	Nbr of shoots
WPM	1.5 BAP	14.2± 2.22^{a}	19.9±5.43^{a}	7.9±2.02^{a}
	1.5 BAP+1NAA	8.9 ±2.50^{b}	2.9±1.3^{b}	2.6±1.11^{c}
	3 BAP	10.5±4.10^{ab}	5.1±2.43^{b}	3.6±0.91^{bc}

1 .5 BAP+1GA3	8.1±4.10[b]	5.2±3.6[b]	5.6±1.95[b]

Means followed by the same letter are not significantly different ($p \leq 0.05$)

Both the concentration and combination of the PGRs significantly affected the number of leaves and shoots per explant and their height. ANOVA also demonstrated a significant interaction between both factors.

Successful organogenesis was observed in WPM medium supplemented with 1.5 mg/L of BAP alone or in combination with NAA and GA3, which produced shoots and leaves. All cultures exhibited satisfactory shooting percentages, and the newly formed shoots exhibited significant elongation. Our most favorable results in terms of leaves and shoot number (7.9 and 19.9) were obtained using the WPM addition with 1.5 mg/L of BAP. Also, BAP at 1.5 mg/L yielded the longest shoot (14.2 cm).

In contrast, WPM supplemented with 1.5 mg/L of BAP combined with 1 mg/L of NAA or GA3 also demonstrated superior results (table 1). The addition of the double concentration of BAP (3 mg/L) to WPM medium also had a significant effect on shoot length (10.5 cm), but it had a negative impact on shoot number (3.6) (table 1).

2.2. Multiplication phase

During the multiplication phase of *Ilex aquifolium*, after one month of cultivation in the WPM medium supplemented with (1.5 mg/L) BAP combined with 1 mg/L NAA, the formation of callus in the basal part of the vitro plants was observed (Figure 1a). These calls hindered both root growth and shoot elongation. When the vitro plants were transferred to the WPM medium containing (1.5 mg/L) NAA, a new formation of roots appeared around the callus (Figure 1b). This represents an indirect rhizogenesis.

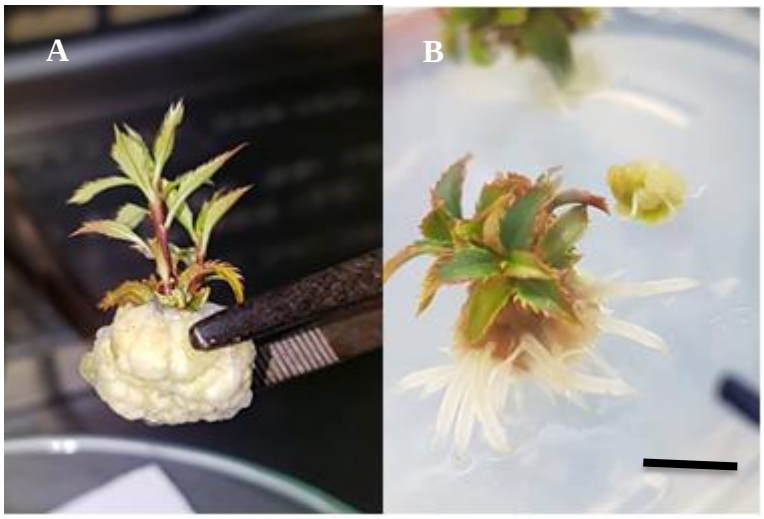

Fig.1. A) Formation of callus in the basal part B) indirect rooting of the vitro plants (b).

2.3. Rooting phase

To test the effect of cultivation media alone or supplemented by different concentrations and combinations of growth hormones on root induction and development, four treatments were performed.

Following the explants' transfer to the tested rooting media, the medium containing 1.5 mg/L of NAA was shown to be the most active, exhibiting a 25% rate at week three and a 100% rate at week eight (table 2). The resulting roots are pale in color and have a short length (Figure 3 a).

However, all growth regulators, namely, have no significant effect on the rooting process for all tested in vitro plants (table 2). After a month, it was shown that media WPM treated with 1 mg/L of NAA or 1 mg/L of IAA did not stimulate the induction of rhizogenesis, but they had a positive impact on the development of the aerial part of the in vitro plants, giving it a bright green color (figure 3b).

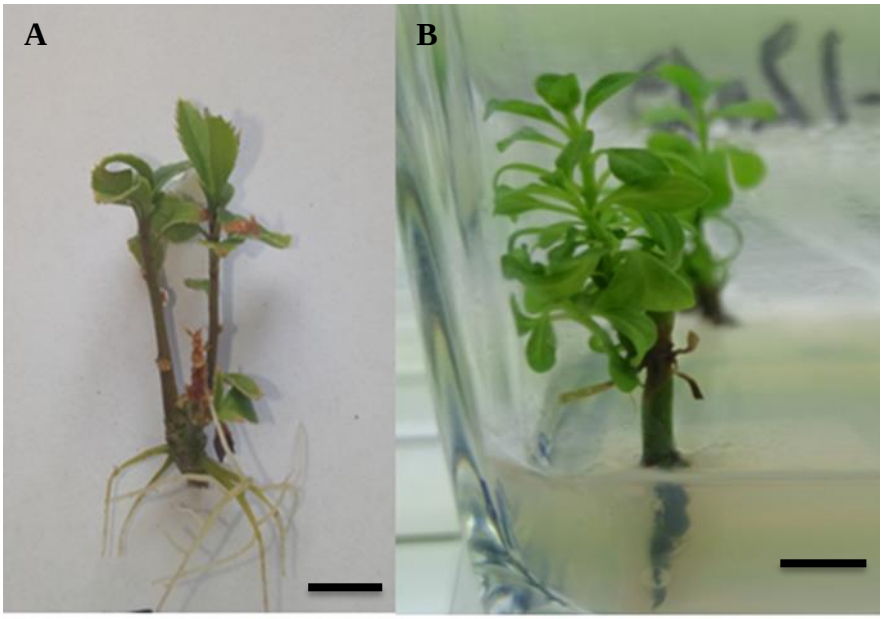

Fig.2. A) Elangation B) rooting of plants (scale bar 1cm).

Table-2. Effect of WPM medium and growth regulators on rooting

Medium (WPM)	Combination (mg/L)				Roots rate after Four weeks (%)	Roots rate after eight weeks (%)	Mean number of roots	Mean length of roots (cm)
	BAP	NAA	IAA	IBA				
M1	1.5	1				0		
M2		1.5			25	100	8.5 ±7.06	0.82 ±0.52

M3		1				0		
M4			1			0		

2.4. Acclimatization phase

The acclimatization of *Ilex aquifolium* L. plants poses another particular challenge. According to this study, the micropropagation of this species through axillary buds required a minimum period of 5 to 6 months to produce *in vitro* plants ready for field transfer. Based on the conducted trials, it was observed that the survival of *in vitro* plants during the *ex vitro* transfer is closely linked to the quality of the root system produced. Consequently, well-rooted shoots were transferred to cultivation pots containing peat and sand (1:1), irrigated with a nutrient solution (WPM/10). The flasks were incubated in a growth chamber for three weeks with a progressive decrease in relative humidity. In vitro plants over 6 months with a healthy root system showed a good survival rate. However, their growth was very slow.

Fig.3. Acclimatization of plants.

3. Discussion

The microprogration approach of *Ilex aquifolium* L. from young seedlings from the germinated zygotic embryos was developed in this work. Growth, morphogenesis, and development of plant tissue culture are mostly governed by the composition of culture media and plant growth regulators (PGRs); the latter are mostly required for growth, morphogenesis, shoot multiplication, and elongation (Pathak et al., 2023). It has been demonstrated that cytokinine, which stimulates the growth of new axillary shoots and results in high rates of multiplication, is crucial for shoot proliferation in hard wood species (Hakim and Dalimunthe, 2022). Results previously reported indicate that in certain wooding species, BAP is the most commonly employed cytokinin for shoot proliferation (Tripathi and Kumari, 2010; Germana et al., 2011; Nagalakshmi et al., 2014).

The reason for BAP's efficacy may be that tissues can digest it more readily than other synthetic growth regulators, or it may be because BAP can stimulate the body to produce natural hormones like zeatin (Malik et al., 2005).

In our study, the concentration of 1.5 mg/L of BAP had a significant effect on the number of shoots (7.9) and leaves (19.9) and the abundance of shoot length (14.2 cm); these numbers were higher than those reported with English holly (Majada et al., 2000), *Ilex dumosa* var. dumosa (Luna et al., 2003), and Ilex paraguariensis A. St. Hil. (Sansberro et al., 1999; Zaniolo and Zanette, 2 001).

The combination of auxines and cytokinines constitutes the most effective group of plant growth regulators for regulating cell division and prolongation as well as for determining morphogenesis (Zuraida et al., 2013).

The analysis of the results of the effect of different hormonal combinations, at different concentrations, on the organogenesis of in vitro regenerated *Ilex aquifolium* stems showed good results. Unlike Majda et al. (2020), the combination of GA3 with BA has a negative effect on the elongation of the stems of *Ilex aquifolium*. Yang et al. (2022) also proved that the addition of GA3 with WPM has an adverse effect on the development of *Populus ussuriensis* Kom. shoots.

The effect of the different media on leaf growth seemed to be very heterogeneous; leaf size was maximum on the medium WPM supplemented with BAP at 1.5 mg/L, followed by the medium WPM supplements by (1.5 mg/l of BAP + 1 mg/L of NAA), then WPM added with (1.5 mg/L of BAP + 0.5 mg// L of GA3). The neoformation of the buds was strongly dependent on the composition of the different mediums. Indeed, the medium WPM + BAP (1.5 mg/L) proved to be most favorable to the development of buds. While the double concentration of BAP (3 mg/L) added to the base medium WPM has a negative effect on neoformation of buds. Our results are promising with those of Majda et al. (2020) on the same species and Luna et al. (2003) on *Ilex dumosa* var. dumosa Reissek.

Adventitious roots initiated from shoots culturing two weeks on WPM medium supplemented with 1.5 mg/L of NAA. The percentage of rooted explants ranged from 25% to 100% (Table 2). Similarly, Majada et al. (2000) reported that roots of English holly formed after 2 weeks of culture. However, rooting of *Ilex dumosa* var. dumosa took 4 weeks (Luna et al., 2003). Rooting of *Ilex glabra* on media supplemented with NAA began to root after three weeks (Sun et al., 2010). Environmental conditions required for *ex vitro* growth of plants are different from those required for *in vitro* cultivation (Hazarika, 2003). In the process of acclimation, excessive water loss from seedlings is a major problem (Hazarika, 2006).

In our work, in vitro regenerated seedlings were acclimatized with a survival rate of 80%; our results are similar to those of Dang et al. (2011) on *Ilex khasiana*.

4. Conclusion

In this study, a successful protocol for the micropropagation of *Ilex aquifolium* L., a major plant, was developed by optimizing growth regulators. These results offer prospects for the conservation and multiplication of the *Ilex aquifolium*, which could increase the plant material available for the production of active compounds from this species.

The results of our organogenesis study demonstrate a significant effect, attributable to the composition of the WPM medium as well as the action of growth regulators, on the bud length, number of leaves and buds produced. The addition of BAP alone or in combination with gibberelline or NAA in the WPM medium has successfully stimulated the proliferation of explants.

WPM medium supplimented with NAA has proved to be the most effective for stimulating root formation in this species in *in vitro* culture. After 4 weeks, all shoots developed roots (100%) in the presence of 1.5 mg/L ANA. Simultaneous rooting of the shoots in substrates also showed promising results, improving the survival rate during the acclimatization phase.

The findings of this study offer evidence that the *in vitro* culture protocol developed can serve as a practical method for both micropropagation and conservation of this endangered species, using indirect and direct organogenesis, as well as for the transfer of plantations grown *in vitro* to the field.

References

Brunu, A. (2011). Sistematica, distribuzione, ecologia e aspetti gestionali delle foreste di tasso (Taxus baccata L.) e agrifoglio (*Ilex aquifolium* L.) in Sardegna.

Dang, J. C., Kumaria, S., Kumar, S., & Tandon, P. (2011). Micropropagation of *Ilex khasiana*, a critically endangered and endemic holly of Northeast India. AoB Plants, 2011, plr012.

Galle, F. C. (1997). Hollies: The Genus Ilex. Portland: Timber Press.

Ge, X., Chu, Z., Lin, Y., & Wang, S. (2006). A tissue culture system for different germplasms of indica rice. Plant cell reports, 25, 392-402.

Germana, M. A., Micheli, M., Chiancone, B., Macaluso, L., & Standardi, A. (2011). Organogenesis and encapsulation of in vitro-derived propagules of Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirius trifoliata* (L.) Raf]. Plant Cell, Tissue and Organ Culture (PCTOC), 106, 299-307.

Hakim, L., & Dalimunthe, A. (2022). Season, basal media and plant growth regulators effect in wood plant in vitro propagation: a comprehensive review. In IOP Conference Series: Earth and Environmental Science (Vol. 1115, No. 1, p. 012051). IOP Publishing.

Hall Publishers, Upper Saddle River, NJ, 915 pp.

Harrington, T. B., & Reichard, S. H. (2007). Meeting the challenge: invasive plants in Pacific Northwest ecosystems, Seattle, Washington, USA, 19-20 September 2006. In Meeting the challenge: invasive plants in Pacific Northwest ecosystems, Seattle, Washington, USA, 19-20 September 2006. (No. PNW-GTR-694). Pacific Northwest Research Station, USDA Forest Service.

Hartmann H. H., Kester D. E., Davies Jr. F. T., Geneve R. L. (2011). Hartmann & Kester's Plant Propagation: Principles and Practices (8th Edition): Prentice

Hazarika, B. N. (2003). Acclimatization of Tissue-cultured Plants. Curr. Sci., 85: 1704-1712.

Hazarika, B. N. (2006). Morpho-physiological disorders in in vitro culture of plants. Scientia horticulturae, 108(2), 105-120.

Hu, C. Y. (1989). Holly (Ilex spp.). In Trees II (pp. 412-427). Berlin, Heidelberg: Springer Berlin Heidelberg.

Ikeuchi, M., Ogawa, Y., Iwase, A., & Sugimoto, K. (2016). Plant regeneration: cellular origins and molecular mechanisms. Development, 143(9), 1442-1451.

Karrfalt, R. P. (2008). Seed testing. The woody plant seed manual. Agriculture Handbook, 727, 97-115.

Luna, C., Sansberro, P., Mroginski, L., & Tarragó, J. (2003). Micropropagation of *Ilex dumosa* (Aquifoliaceae) from nodal segments in a tissue culture system. Biocell, 27(2), 205-212.

Majada, J. P., Sánchez-Tamés, R., Revilla, M. A., & Casares, A. (2000). Micropropagation of *Ilex aquifolium* L. In Vitro Cellular & Developmental Biology-Plant, 36, 521-526.

Malik, S. K., Chaudhury, R., & Kalia, R. K. (2005). Rapid in vitro multiplication and conservation of Garcinia indica: a tropical medicinal tree species. Scientia Horticulturae, 106(4), 539-553.

Nagalakshmi, M., Vishwanath, S., & Viswanath, S. (2014). Adventitious shoot regeneration from hypocotyls of Wrightia arborea (Dennst.) Mabb.: an endangered toy wood species. Journal Cell and Tissue Research, 14, 4339-4344.

Pathak, A., Haq, S., Meena, N., Dwivedi, P., Kothari, S. L., & Kachhwaha, S. (2023). Multifaceted Role of Nanomaterials in Modulating In Vitro Seed Germination, Plant Morphogenesis, Metabolism and Genetic Engineering. Plants, 12(17), 3126.

Sansberro, P., Rey, H., Mroginski, L., & Collavino, M. (1999). *In vitro* plant regeneration of Ilex paraguariensis (Aquifoliaceae). In Vitro Cellular & Developmental Biology-Plant, 35, 401-402.

Shahzad, A., Parveen, S., Sharma, S., Shaheen, A., Saeed, T., Yadav, V., & Upadhyay, A. (2017). Plant tissue culture: applications in plant improvement and conservation. Plant Biotechnology: principles and applications, 37-72.

Sun, Y., Zhang, D., & Smagula, J. (2010). Micropropagation of *Ilex glabra* (L.) A. Gray. HortScience, 45(5), 805-808.

Sun, Y., Zhang, D., & Smagula, J. (2010). Micropropagation of *Ilex glabra* (L.) A. Gray. HortScience, 45(5), 805-808.

Tripathi, M., & Kumari, N. (2010). Micropropagation of a tropical fruit tree Spondias mangifera Willd. through direct organogenesis. Acta Physiologiae Plantarum, 32, 1011-1015.

Yahi, N., Djellouli, Y., & de Foucault, B. (2008). Diversités floristique et biogéographique des cédraies d'Algérie. Acta Botanica Gallica, 155(3), 389-402.

Yang, S., Liu, R., Li, W., Jing, Y., Pak, S., & Li, C. (2022). Rapid and efficient regeneration of Populus ussuriensis kom. From root explants through direct de novo shoot organogenesis. Forests, 13(5), 806.

Zaniolo, S. R., & Zanette, F. (2001). Micropropagation of the erva-mate through culture of nodal segments.

Zuraida, A. R., & Shukri, M. A. Ayu Nazreena O, Zamri Z (2013). Improved micropropagation of biopesticidal plant, Pelargonium radula via direct shoot regeneration. Am J Res Commun, 1(1), 1-12.