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Fabiana imbricata Ruiz et Pav. micropropagation

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Abstract

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The effects of growth regulators on shoot development and rooting have been studied in *Fabiana imbricata* Ruiz et Pav. micropropagation. Plants were cultured *in vitro* on media with 0.1, 0.25 and 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and 0.01 mg l^{-1} indole-3-butyric acid (IBA). In all the treatments similar values for the mean height of the plants and 100% rooting were established. Data analysis showed that within 28 days the proliferation value in the hormone-free variant was 5.04±0.68 compared to the treatment with 0.5 mg l^{-1} BAP and 0.01 mg l^{-1} IBA (5.64±0.70), where symptoms of hyperhydricity occurred. Application of 0.3% activated charcoal (AC) influenced the growth and development of the plants positively, resulting in almost two-fold higher proliferation (9.04±0.54), overcoming this physiological disorder. Moreover, the optimized propagation system allowed the organogenesis of both, shoots and roots simultaneously, as approach to accelerate the micropropagation process and reduce production costs. Although the percentage of surviving plants was dependent on root system morphology, the lack of growth regulators in the last subculture before adaptation could be a useful prerequisite for the hardening of process, resulting in successful plant survival.

Keywords: Fabiana imbricata Ruiz et Pav.; activated charcoal; micropropagation

Introduction

Plant tissue culture is a complex of methods for producing plantlets from single cells, tissues or organs isolated from the plant following these techniques**,** providing an opportunity for rapid mass propagation, induction of genetic diversity, creation of new cultivars and fundamental biological studies (George et al., 2008). Propagation of some ornamental plants has increased tremendously due to the demand for them as cut flowers, in addition to their use for interior and exterior landscaping purposes (Çiğ & Başdoğan, 2015).

Successful *in vitro* propagation of ornamental plants is now being used for commercialization. Many commercial laboratories and national institutes worldwide use *in vitro* culture systems for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations, and for secondary metabolite production (Rout et al., 2006, Nazki et al., 2018).

The medicinal plant *Fabiana imbricata* Ruiz et Pavon, also called Pichi Pichi or Palo Pichi belongs to the family of Solanaceae. It is a resinous bush typical of Andean region, originally from Chile and covering Argentinian-Chilean Patagonia. Plants can also be found in Peru, Bolivia and Brazil. This shrub is found in arid and stony lands near rivers or on road verges. The plant grows wildly, and its evergreen tiny, needle-like, dark green leaves (to 5 mm) are used to produce *Fabiana* essential oil by steam distillation. The upright form *F. imbricata* f. *violacea*, bearing masses of pale violet flowers, has ornamental value in different gardens. Its branches transform into plumes of violet tubular flowers in late spring – the plant flowers from November to January in the southern hemisphere and from May to June in the north-

ern hemisphere. The flowers are small, trumpet-shaped, and vary in colour from white to purple. The fruits are oval capsules about 1cm or less in length (Rätsch, 1998).

F.imbricata Ruiz et Pav. is traditionally used in Chilean folk medicine to treat diseases of the urinary tract and kidneys. It is a potent diuretic which also promotes digestion when prepared as a tea. *F. imbricata* has a long history of use in the treatment of general diseases as well. A mother tincture is occasionally used in homeopathy for the cure of the liver and urinary system, as well as a general tonic. The water/alcohol extract is beneficial as antiseptic (Rätsch, 1998). Fabiana essential oil is principally used for its diuretic and urinary tract antiseptic properties. It is known to have anti-rheumatic properties and effect on pulmonary diseases, but also it has application in natural cosmetics (Marquez & Diana, 2004; Alonso & Desmarchelier, 2006; Festy, 2016).

The chemical composition of the plant *Fabiana imbricata* Ruiz et Pav. is less studied in details. A particular interest is paid to the investigation of the metabolite profiles as a basis for further isolation and characterization of valuable secondary metabolites. It has been reported that the principal components of the secondary metabolite mixture in the crude drug are rutin, coumarin scopoletin, oleanic acid, and several sesquiterpenoids (Schmeda-Hirschmann et al., 2004).

Fabiana imbricata is propagated by seeds which should be pre-germinated and potted once they are seedlings. It is grown as an ornamental plant inside and in open gardens in areas that rarely see frost (Rätsch, 1998). The plant has a high vegetative propagation potential *in vivo* and *in vitro,* whereas the multiplication *in vitro* is faster, enabling rapid multiplication and valuable secondary metabolite production (Schmeda-Hirschmann et al., 2004). Shoot regeneration is possible also from callus culture (Pinker et al., 2008).

Our scientific interest was directed at the study of micropropagation and further characterization of the metabolic activities of *in vitro* plants as a source of subsequent callus and cell cultures, generating valuable biologically active substances.

Materials and Methods

Initial explants were taken from green shoots of potted plants *Fabiana imbricata f.violacea* grown indoors. Isolated cuttings (2 cm length) were first washed in tap water with detergent for 30 min and sterilized by treatment with 0.1% solution of mercuric chloride ($HgCl_2$) for a minute, followed by five two-minute washes with sterile water. Explants were placed individually in glass tubes with A0 medium (Table 1) and cultivated in a growth chamber with a temperature of 24±1°C, light intensity of 3000 lx and 16/8h photoperiod.

Propagation MS-based media (Murashige & Skoog, 1962) were supplemented with 30g l^{-1} sucrose, 6g l^{-1} agar, and pH = 5.7. The subsequent cultivation of the developed explants was performed on media with various concentrations of growth regulators (Table 1) in glass vessels (volume 180 ml) containing 30 ml nutrition medium and duration of the subculture of four weeks.

All investigated treatments consisted of five replications, each containing five explants, and the average data analysis was based on three independent experiments. The following indicators were counted: mean number of shoots per explant, mean plant height, mean number of roots and mean root length. The effect of the propagation media was studied dynamically by data collection on days 14, 21 and 28, and analysed by standard biometrical methods.

Adaptation *ex vitro* and acclimatization of the plants obtained were performed in a growth chamber with gradually decreasing atmospheric humidity, temperature of 22±1°C and 16/8 h photoperiod. A mixture of peat-perlite (3:1) with the addition of sand (50%) was used as a substrate for transplanting in pots. The percentages of surviving plants cultivated on media A0 and A33 were compared on days 7, 14, 21 and 28.

Results and Discussion

Following the sterilization procedure described over 98% of the explants demonstrated development on A0 medium. Formation of shoots and roots was observed after four weeks of cultivation.

Effect of the growth regulators concentration

Initially, the *in vitro* obtained plants were grown on A0 medium in two subcultures. Then the influence of the growth regulators concentration was compared in the proliferation stage. Results counted on day 14 showed that all explants

Table 1. Concentration of the growth regulators in the propagation media (mg l^{-1})

Culture medium / Additions	A0	A1	A25	A3	A33
BAP	0	0.1	0.25	0.5	0.5
IBA	0	0.01	0.01	0.01	0.01
Activated charcoal (AC)	0	0	0	0	3000

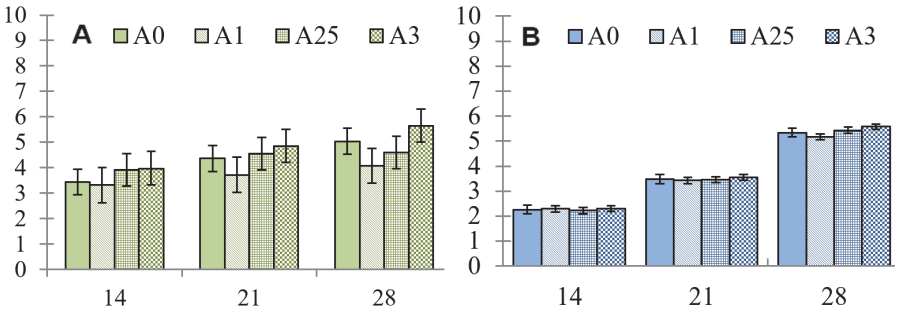

Fig. 1. Effect of growth regulators on *Fabiana* micropropagation as mean number of shoots per explant (*A*) and mean plant height (*B*) on days 14, 21 and 28, (±SE)

grown on hormone-free medium proliferated by an average of 3.44±0.51 shoots per explant, which is similar to the variants with the addition of growth regulators (Fig. 1A). On day 28, the proliferation value in variant A0 was 5.04±0.68 compared to variant A3 – 5.64±0.70. Surprisingly, the explants cultivated on media A1 and A25, enriched with growth regulators, demonstrated lower proliferation than those grown on the hormone-free medium A0.

Comparing the efficiency of the studied proliferation media, variant A3 demonstrated the highest proliferation capability, but the concentration of the applied cytokinin (BAP) caused development of shoots with symptoms of hyperhydricity (vitrification). Data analysis of the mean plant height indicator showed that on day 14 the plants grew to 2.2 cm, reaching over 5 cm on day 28 (Fig. 1B). In all the treatments, similar values for the mean plant height were established. Schmeda-Hirschmann et al., (2004) reported the development of a rapid *in vitro* propagation system for *Fabiana imbricata* leading to the formation of shoots, calli, roots, cell suspensions and plantlets. In their study, the shoots derived from nodal sections were multiplied by branching new axillary buds by means of the temporary immersion system (TIS) with an average shoot length of 5 cm.

Effect of the addition of AC on proliferation

An optimized proliferation medium (A33) was tested to overcome the physiological disorder vitrification. It had the same composition as A3 (Table 1), but with activated charcoal addition (0.3 %).The application of activated charcoal positively influenced the growth and development of the plants, resulting in almost two-fold higher proliferation and absence of hyperhydricity. Cultivation of the explants on the A33 medium was characterized with the formation of 9.04 ±0.54 shoots per explant in comparison to A3 (5.64±0.70) on day 28 (Fig. 2A). The results for the mean plant height indicator showed similar values for both variants again (Fig. 2B).

The use of AC as a culture component for adsorption of toxic plant metabolites is known, but according to Pan and van Staden (1998) it can also adsorb high concentrations of the growth regulators BA, IAA, IBA, NAA and Kinetin (Weatherhead et al., 1978) in both liquid and solid media (Nissen & Sutter, 1990).

Regarding the possible influence of AC on hyperhydricity, some stimulative, unclear, or inhibitory effects on the multiplication of various plants were discussed (Debergh et al., 1981; Pan and van Staden, 1998). Moreover, Druart and De Wuif (1993) suggested that the extent of sucrose hydrolysis in tissue culture media containing activated charcoal is

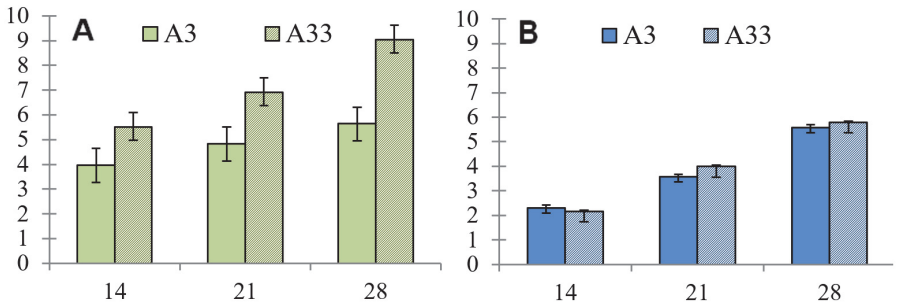

Fig. 2. Effect of activated charcoal addition on *Fabiana* micropropagation as (A) mean number of shoots per explant, and (B) mean plant height on days 14, 21, and 28, (±SE)

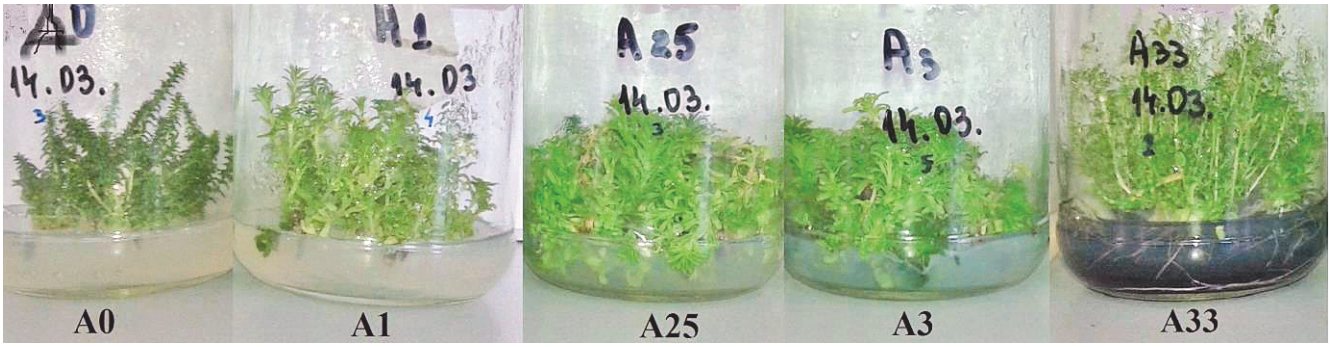

Fig. 3. Characterization of the plants cultivated on studied media on day 28

Fig. 4. Plant development on culture media A0 и A33

proportional to the hydrogen ion concentration and pH, and such interactions also should be taken into consideration.

Characterization of the plants cultivated on proliferation media

Growth regulators concentration was essential for the morphogenesis and development of the plants (Fig. 3). Plants cultivated on A0 were characterized with a compact well-formed shrub, dark green leaves and short roots. Medium A1 induced shoots with different lengths, light green stems, and formation of small roots. Both media, A25 and A3, demonstrated high proliferation but associated with shortened internodes and visible symptoms of vitrification. The growth behaviour and plant development on A33 were strongly influenced by the addition of activated charcoal and characterized by the formation of symmetrically shaped shrubs and elongated roots compared to those grown on variant A0 (Fig. 3 and Fig. 4).

According to Cheruvathur et al. (2010), the beneficial effects of activated charcoal on the *in vitro* tissue response may result from the darkening of the culture medium for multiple root induction or the adsorption of plant growth regulators and other organic compounds.

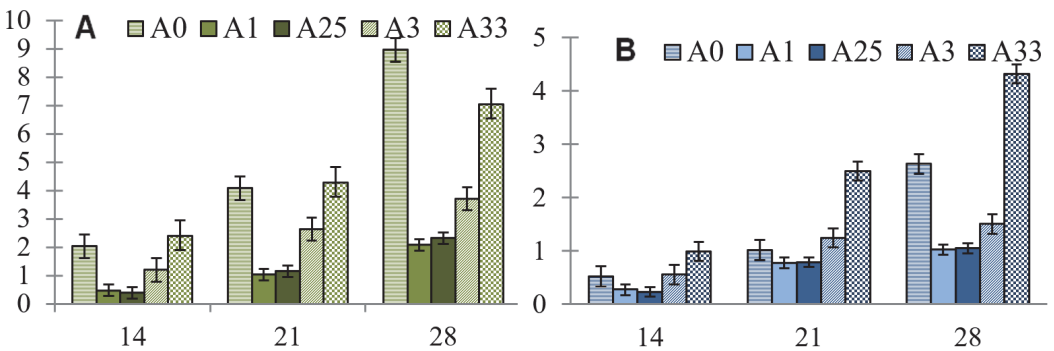

Fig. 5. Rooting capacity as mean root number (A), and mean root length (B) of plantlets cultivated on different propagation media on days 14, 21 and 28, (±SE)

Rooting

During the stage of multiplication, all explants (100%) cultivated on different proliferation media produced roots. Data obtained on days 14, 21 and 28 confirmed that A0 and A33 were the most efficient media, inducing the formation of a high mean number of roots and mean root lengths (Fig. 5). At the end of the culture, the highest values of mean root numbers (8.96 and 7.04) recorded for variants A0 and A33 respectively (Fig. 5A).

However, the data obtained for the mean root length indicator (Fig. 5B) demonstrated that placement on A0 resulted in the formation of short and thick roots compared to variant A33, where slim, long and tender roots developed (Fig. 4).

The three initiation media tested by Schmeda-Hirschmann et al., (2004) in the experiment with *Fabiana imbricata* also led to rhizogenesis. Root formation of single shoots mostly occurred after one month in the presence of IAA, IBA or NAA used alone or in combinations at levels of 0.25 – 1.0 mg l^{-1}. Under these conditions, rooting occurred in 41.2-64.7% of explants, with the highest rate of 5.6 roots per explant (Schmeda-Hirschmann et al., 2004).

The propagation medium with cytokinine/auxin ratio 50:1 applied by us demonstrated efficiency in the development of the *Fabiana* plants and tolerated shoot and root formation at the same time. Such growth behaviour could be explained with the specific endogenous content of growth regulators in the plant species, allowing the organogenesis of both, roots and shoots simultaneously, on the same medium. A similar morphogenic response was described in the medicinal plant *Wedelia Chinensis* as an approach to accelerate micropropagation and reduce production costs (Agarwala et al., 2010). Contrary, based solely on the application of auxins a coincidence of shoots and roots of different grape cultivars and rootstocks was reported (Yancheva et al., 2018). Normally, the type of *in vitro* response to the growth regulators applied could be explained by the natural specificity of the particular plant species, again confirming the strong dependence of organogenesis on genotype.

Adaptation and plant survival

The results presented in Fig. 6 show the influence of the cultivation medium on the adaptation efficiency as a percentage of surviving plants.

Following the acclimatization procedure, the plants grown on medium A0 demonstrated the highest rate (80%) of survival. In comparison, the percentage of vital plants originating from variant A33 decreased from 70% on day 7 to 30% on day 35 (Fig. 6 and Fig. 7). Based on these results we suggest that the omission of the growth regulators in the last subculture before adaptation could be a useful prerequisite for plant hardening, resulting in successful survival.

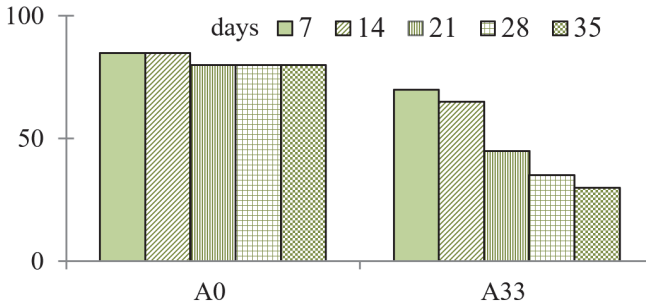

Fig. 6. Influence of the rooting medium on adaptation and plant survival

Fig. 7. Survival of plants following 35 days of adaptation

Activated charcoal is pure carbon that has been specially processed to adsorb a wide variety of chemical compounds and gases (Sáenz et al., 2010). As an inexpensive substance, AC has been commonly used in tissue culture media to improve growth and development. Many research studies have shown that the addition of AC often has a stimulative effect on the growth and organogenesis of different plants (Mensuali-Sodi et al., 1993). Reviewing the application of charcoal *in vitro* in different plants, Pan and van Staden (1998) concluded that its addition to culture medium may promote or inhibit *in vitro* growth, depending on the species and tissues used. The effects of activated charcoal may be attributed to establishing a darkened environment; adsorption of undesirable/inhibitory substances; adsorption of growth regulators and other organic compounds, or the release of growth promoting substances present in or adsorbed by activated charcoal (Pan & van Staden, 1998). According to Sáenz et al., (2010), activated charcoal may also determine whether an endogenous auxin/cytokinin level that is adequate for root production may be reached.

Conclusions

The present investigation showed the development of an optimized composition of the culture medium for *Fabiana imbricata* micropropagation. The application of AC in the micropropagation system reported here had a stimulative ef-

fect on micropropagation, first, on proliferation efficiency by increasing the mean number of shoots, average shoot length and eliminating hyperhydricity, and second, on rhizogenesis – as mean root length. Moreover, the specificity of the plant species determined the *in vitro* behaviour and allowed the formation of both shoots and roots simultaneously, as a useful approach towards acceleration of micropropagation and reduction of production costs. Further characterization of the metabolic activities of *in vitro* plants could be a prerequisite for subsequent development of callus and cell cultures, generating valuable biologically active substances.

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