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ORIGINAL ARTICLE

In vitro Culture of Immature Embryos of Mastic Tree (*Pistacia lentiscus* L.)

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A R T I C L E I N F O

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A B S T R A C T

Immature lentisk (*Pistacia lentiscus* L.) seeds from plants grown in Liguria (Italy) were collected and surface sterilized with ethanol (70%) and then NaOCl (1%). The outer pericarp of the immature fruits was removed and shells were opened. Enhancement of Embryo germination was examined through the use of five sucrose concentrations (0, 15, 30, 45, and 60 g L^{-1}) combined with five concentrations of GA_3 (0, 0.1, 0.3, 0.5, and 0.7 mg L^{-1}). After 14 days, the germination frequency was detected and after 16 days, seedling development was evaluated. The embryo development into plantlets increased up to 54.37% when 45 g L^{-1} of sucrose was supplied; at this concentration, all the parameters, such as plantlet weight, height, and root length were high, while at 60 g L^{-1} of sucrose, the plantlets showed many lateral root formations. The root shape was completely different among the treatments. The percentage of embryo germination, plantlet weight and height, root length, and main lateral root number were enhanced when GA_3 was used in the concentration of 0.7 (mg L^{-1}). No rooting at all was observed when GA_3 was used in a concentration of 0.3 or 0.5 (mg L^{-1}) or medium without GA_3. This research increases the possibility to obtain a massive number of plants to be used as rootstock for pistachio cultivation in difficult soils.

Introduction

Lentisk (*Pistacia lentiscus* L.), also known as "mastic tree", is a widespread ornamental shrub in different Mediterranean regions like Italy (Ak and Parlakcı, 2009, Mahmoudi Meimand *et al.*, 2020 (a)); it is an evergreen plant with dark green foliage, red to dark berry fruits which can be used for medicinal purpose and it represents the main source of a unique resin named mastic (Mascarello *et al.*, 2007). Lentisk is a dioecious plant with separate male and female stocks, in which females produce lower levels of mastic than males (Acar, 1988). It is well adapted to many severe climatic conditions such as drought, saline, and calcareous soil, for this it is considered as the main pistachio rootstock (Zohary, 1952; Correia and Catarino, 1994; Ladd *et al.*, 2005; Mascarello *et al.*, 2007; Mahmoudi Meimand *et al.*, 2020 (a)). Lentisk plant naturally propagated by seed with high differences in germination rate among different genotypes as well as an increase of genetic variability (Mascarello *et al.*, 2007). Therefore, when seeds are used for propagation, the useful traits of parents are diminished in the next generations because of genetic alterations (Yildirim *et al.*, 2019). Furthermore, when female lentisk trees are obtained (about 50%) by seed propagation, they are not productive for harvesting mastic gum (Onay *et al.*, 2016). In addition, this plant has serious propagation difficulties, such as parthenocarpy and ovary abortion (Mascarello *et al.*,

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2007). Similar to other plants of the Anacardiaceae family, the vegetative propagation by cutting is so hard because of the poor induction of adventitious roots (Mahmoudi Meimand *et al*., 2020 (b)).

The *in vitro* culture could be a beneficial tool to avoid problems on vegetative propagation and to obtain homogeneous material (Fascella *et al.,* 2004; Ruffoni *et al.,* 2004; Mahmoudi Meimand *et al*., 2020 (b)). Despite this but, efforts on the improvement of micropropagation protocols for lentisk had been insufficient until 2004 (Yildirim *et al.*, 2019). Among different *in vitro* culture techniques, embryo rescue has been widely focused on promoting weak or immature embryo development (Collins and Grosser, 1984; Ebrahimzadeh *et al*., 2021). Embryo rescue can be done with or without embryo excision, in some cases, it has not been technically possible to remove the embryos from the ovules, so they are cultured without embryo excision (Sharma *et al.,* 1996). *In vitro* culture of the immature embryo has been frequently applied in many fruit crops, including peach (Anderson *et al.,* 2002), persimmon (Leng and Yamamura, 2006; Yamada and Tao, 2007; Hu *et al.,* 2013), apple (Dantas *et al.,* 2006), citrus fruits (Viloria *et al.,* 2005; Xie *et al.,* 2014), banana (Bakry 2008; Uma *et al.,* 2011), mango (Krishna and Singh, 2007), grape (Li *et al.,* 2014) and walnut (Vahdati *et al.*, 2006; Grouh *et al.*, 2011) for various purposes such as recover maternal haploids and progeny from intraspecific hybridizations. Embryo rescue has been a very successful procedure in overcoming germination barriers in wide hybridization programs (Collins and Grosser, 1984). Moreover, embryo rescue techniques have also been used to overcome seed dormancy (Collins and Grosser, 1984; Ramming, 1990; Gribaudo, 1993; Sharma *et al.,* 1996). Based on previous reports, it was noticed that different basal media, nitrogen source and concentration, sucrose and different hormones type and levels, temperature, and light had significant effects on *In vitro* culture of immature embryo success (Xie *et al.,* 2014; Li *et al.,* 2014). Sharma *et al*. (1996) proved that embryo rescue success depends on the embryo maturity stage and media composition. It is reported that sugar and gibberellic acid concentration play a key role in embryo germination (Guo *et al.,* 2004). Sugar, as a vital compound known as an osmotic stabilizer as well as a carbon source, is a fundamental compound in culture media for the development of embryos (Sharma *et al.,* 1996). Among different sugar types, sucrose is usually used for embryo cultures, with a concentration ranging from 10 to 60 g L^{-1}. High sucrose concentrations were often applied for culturing immature embryos (Emershad and Ramming, 1994; Pommer *et al.,* 1995; Sharma *et al.,* 1996; Bharathy *et al.,* 2003, 2005; Nookaraju *et al.,* 2007; Tian *et al.,* 2008; Tang *et al.,* 2009; Guo *et al.,* 2011; Ji *et al.,* 2013) with different effect and high osmotic potential in the medium prevents precocious germination of embryos (Amemiya, 1964; Niederwieser *et al.,* 1990; Pecket and Selim, 1965; Sharma *et al.,* 1996). According to literature, a medium sucrose concentration between 40 and 50 g L^{-1} was suitable to enhance embryo germination and subsequent plant formation (Valdez and Ulanovsky, 1997; Wakana *et al.,* 2003; Xu *et al.,* 2005; Bharathy *et al.,* 2005; Valdez, 2005; Nookaraju *et al.,* 2007; Tian *et al.,* 2008; Tang *et al.,* 2009; Guo *et al.,* 2011). Furthermore, sugar and plants phytohormones, especially gibberellic acid had also been frequently tested together in embryo culture media (Okamoto *et al.,* 1993; Aguero *et al.,* 1996; Burger and Goussard, 1996; Yamashita *et al.,* 1998; Qi and Ding, 2002; Liu *et al.,* 2003; Wakana *et al.,* 2003; Ebadi *et al.,* 2004; Guo *et al.,* 2004; Yang *et al.,* 2007; Sun *et al.,* 2011; Singh *et al.,* 2011; Koh and Oh, 2013). In many studies, the addition of GA_3 (0.4 or 0.5 mg L^{-1}) to the medium enhanced seed or embryo germination and development (Yamashita *et al.,* 1998; Singh *et al.,* 2011; Ji *et al.,* 2013; Tombegavani *et al*., 2020; Sappalani *et al*., 2021). Guo *et al*. (2004) reported that the best result for grape embryo rescue occurred with the addition of gibberellic acid (GA_3 at 0.5 mg L^{-1}). Nevertheless, other authors reported that some

phytohormones, such as GA_3, have no significant effect on embryo rescue of seedless grapes (*Vitis vinifera* L.) and of tetraploid grapes (*Vitis vinifera* and *V. complex*) (Burger and Goussard, 1996; Wakana *et al.*, 2003). According to our literature review, there is no detailed research and sufficient information for *In vitro* culture of the immature embryo of Lentisk plant, the current study aimed to define an optimized protocol for lentisk embryo rescue procedure because this plant has main propagation problems, which known as ovary abortion. Furthermore, to offer a new alternative to the breeders for obtaining plants from immature seed with high efficiency to be used as the rootstock of *Pistacia vera* for cultivation in unsuitable soils or *Pistacia* breeding programs.

Materials and Methods

General culture conditions and plant material

Immature seeds were collected from lentisk plants grown in wild conditions, in San Lorenzo, Liguria region, Italy, 163 meters above sea level. The lentisk fruit consists of a rather small kernel enclosed in a thin, relative hard shell surrounded by a fleshy green hull when it is unmatured. The nuts were sterilized according to the following protocol, pre-treatment with ethanol (70%) for 30 seconds followed by three times washing with distilled water, the treatment with NaOCl (1%) for 30 min and finally washing three times by distilled sterilized water. The external pericarp of the immature fruits was removed and shells were opened. The immature embryos were placed on Petri dishes containing a semi-solid MS (Murashige and Skoog, 1962) medium composed of macro and microelements and vitamins, gellified by agar 8 g L^{-1}. This medium was used as control and several combinations of Gibberellic acid (GA_3) and sucrose were taken into account.

Statistical analysis

Statistical analysis was performed by using SAS Statistics version 9.4 software package. Experiments were carried out in a factorial design. Five sucrose concentrations involved (0, 15, 30, 45 and 60 g L^{-1}) and five concentrations of GA_3 (0, 0.1, 0.3, 0.5 and 0.7 mg L^{-1}) were combined. For each combination, 3 Petri dishes were taken into consideration and 6 embryos/Petri dishes were cultured. The Petri dishes were placed in a growth room, at 24°C ±2, in dark conditions for two weeks. After 14 days, data on the percentage of germinated embryos were recorded, and shoot length, plant weight, length of the main root, and the number of lateral roots were measured and counted after 30 days. Data were subjected to analysis of variance (ANOVA) and means were compared using Duncan's test ($P \leq 0.01$ and 0.05), before media comparison, the percentages were transformed angular values.

Results

Immature embryo germination

After 14 days, in the absence of sugar, the immature embryos did not germinate (Fig. 1a). The sugar alone, at any concentration, is not enough to induce germination at all; only in combination with GA_3 embryo induction and development can be appreciated. Initially, at 0.1 mg L^{-1} GA_3, the development percentage was low (7.5) in the case of 15 and 30 g L^{-1} of sucrose and increased (30%) at 45-60 g L^{-1}. The increase of the GA_3 concentrations guaranteed higher germination percentages, but the combined effect of sucrose is still evident. The significantly better germination percentage occurred at 60 g L^{-1} sucrose and 0.7 mg L^{-1} GA_3 ($p \leq 0.01$) (Fig. 1); the analysis of the factors showed that the sugar is essential for the embryo germination and directly related to the concentration up to 45 g L^{-1} with a slice decrease at 60 g L^{-1} non statistically evidenced (Fig. 1a). The GA_3 level is important for germination; all concentrations over 0.3 mg L^{-1} permitted a mean germination percentage over 50% without statistical difference among levels (Fig. 1b).

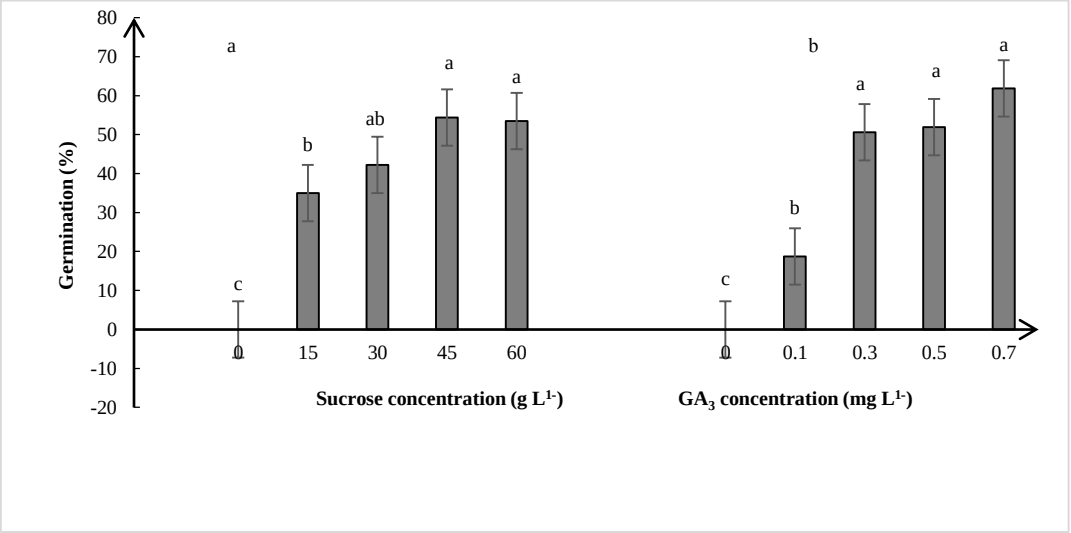

Fig. 1. Germination percentage of immature embryos cultured onto increasing levels of sucrose (a) and GA_3 (b), after 14 days. For each treatment, the same letters mean no significant differences at (P≤0.01 and 0.05).

Morphological parameters

A significant effect of sugar (p≤0.01), and GA_3 (P≤0.01) were detected in the main morphological parameters during the lentisk embryo rescue (Fig. 2.). The weight and the total length (aerial part plus main root) of the seedlings, recorded after 30 days from sowing, showed differently the influence of the presence of sugar in the medium. The weight of the plantlets seemed not to affect by the carbohydrate concentration (Fig. 2) whether the plants were the highest in the presence of 45 g L^{-1} of sucrose (Fig. 2). On the contrary, the evaluation of the data related to the GA_3 concentration showed the effect of the values higher than 0.1 mg L^{-1}, resulting in higher weight and length (Fig. 3).

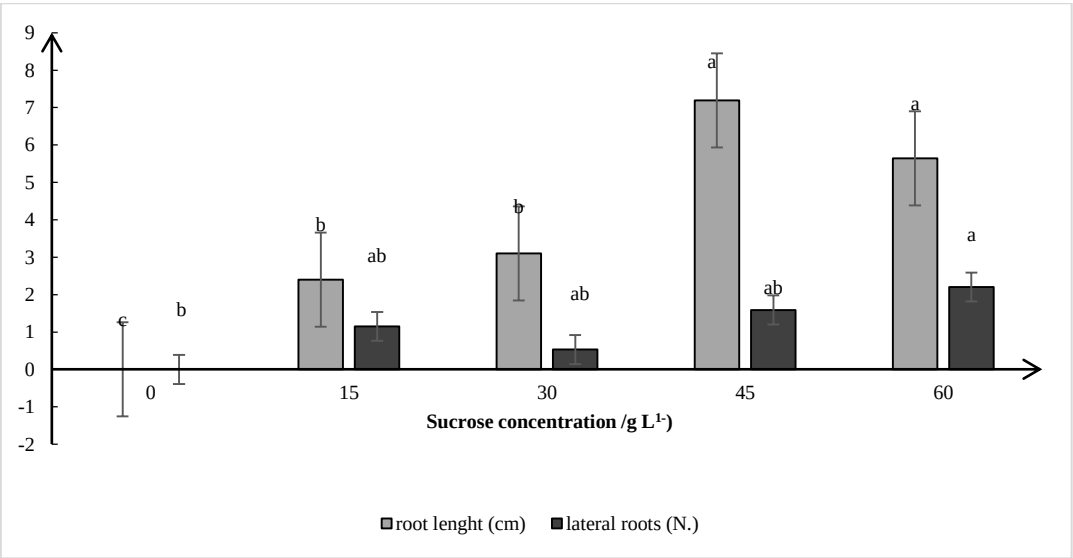

Fig. 2. Plantlets weight (g) and height (cm) cultured onto increasing levels of sucrose, after 30 days. For each parameter, the same letters mean no significant differences at (p≤ 01).

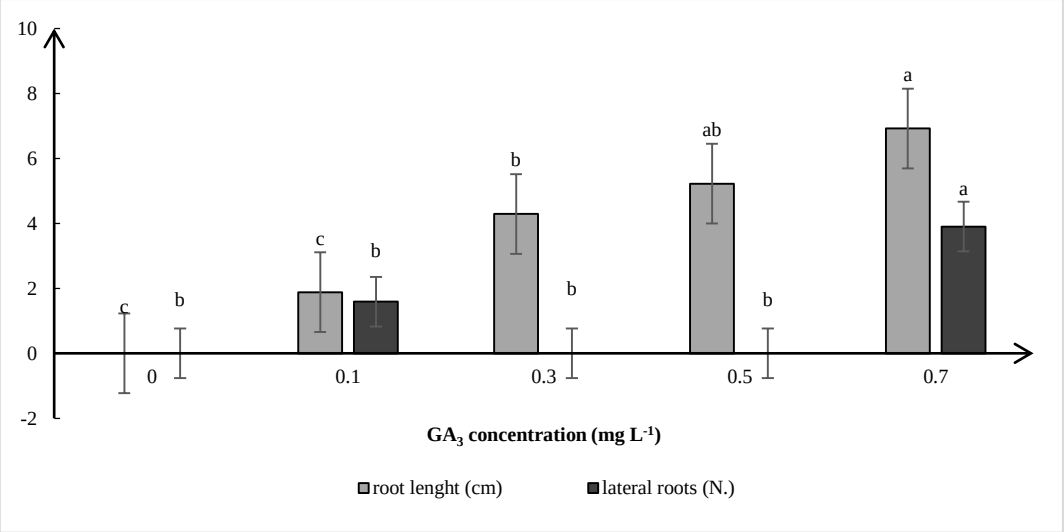

Fig. 3. Plantlets weight (g) and height (cm) were cultured onto increasing levels of GA_3, after 30 days. For each parameter, the same letters mean no significant differences at ($p \leq 01$).

Rooting

All the embryos showed root development with, at least, along the main root. The differences in length of the main root are summarized in Fig. 4a and b; at 45 and 60 g L^{-1} of sucrose, the roots grew more than 5 cm, as statistically evidenced. The length of the main root was directly related to the increasing concentrations of the GA_3 (Fig. 4, b). Sometimes, and independently from the culture medium, lateral adventitious root emerged from the main one. It was possible to evidence a relationship between plant regulators combination and root shape, as shown in Fig. 5. The lowest sucrose (15 g L^{-1}) and GA_3 concentration (0.3 mg L^{-1}) added to the medium were not sufficient to ensure plantlets were ready to be acclimatized; in fact, any root induction was detected (Fig. 5d). The best explants, with a proportional shape between aerial part and root apparatus, was observed when the highest level of sucrose (60 g L^{-1}) and GA_3 (0.7 mg L^{-1}) were supplied; in this case, it was possible to observe many lateral roots and expanse leaves, useful parameters related to further acclimatization (Fig. 5c).

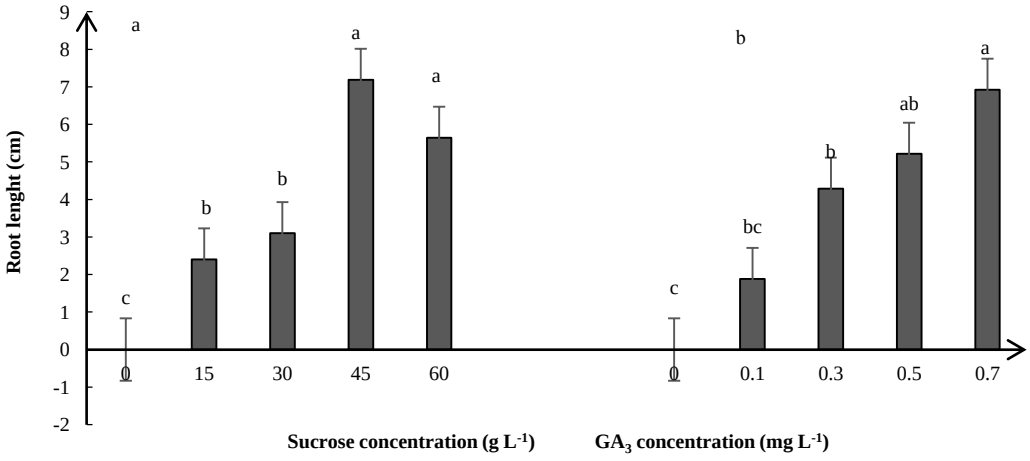

Fig. 4. Root length (cm) related to increasing concentrations of sucrose (g L^{-1}) and GA_3 (mg L^{-1}) for each treatment, the same letters mean no significant differences at ($p \leq 01$).

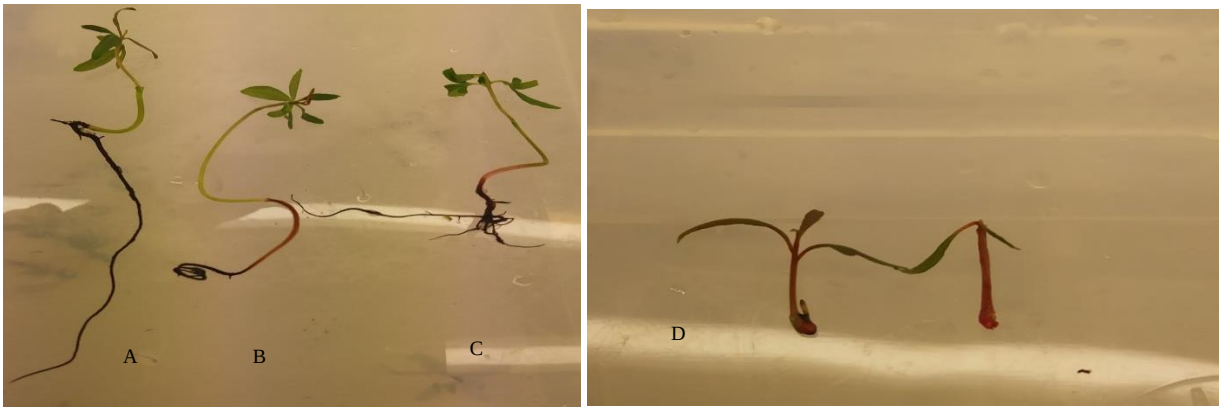

Fig. 5. Different plantlet shapes and behavior related to culture media: a) MS + sucrose (45 g L^{-1}) and GA_3 (0.7 mg L^{-1}), (long root); b) MS + sucrose (30 g L^{-1}) and GA_3 (0.3 mg L^{-1}), (round and Spring-like; c) MS + sucrose (60 g L^{-1}) and GA_3 (0.7 mg L^{-1}), (with more hairy roots); d) MS + sucrose (15 g L^{-1}) and GA_3 (0.3 mg L^{-1}), (without root induction)

Discussion

The sugar alone, at any concentration, is not enough to induce germination at all; unless in combination with GA_3, so the use of sucrose in middle-level concentration incorporate with GA_3, has increased the development of embryos into plantlets. The weight of the plantlets seemed to be indifferent to the carbohydrate concentration. The plants were the highest in the presence of 45 g L^{-1} of sucrose. The influence of sugar was also observed on main lateral root formation and root shapes and also the main root length (cm). The effects of sucrose concentrations on the development of embryos and the growth of plants *in vitro* have been reported by many researchers. Sharma *et al*. (1996) defined sucrose as a main osmotic stabilizer and a carbon source in culture media for the development of plant embryos. Our result demonstrated higher sucrose concentration has increased the development of embryos into plantlets, similar results have also been reported by Emershad and Ramming (1994); Pommer *et al*. (1995); Sharma *et al*. (1996); Bharathy *et al*. (2003, 2005); Nookaraju *et al*. (2007); Tian *et al*. (2008); Tang *et al*. (2009); Guo *et al*. (2011) and Ji *et al*. (2013) which stated that higher sucrose concentration often applied for culturing immature plant embryos. In agreement with our results, previous reports showed that the high osmotic potential of the medium prevents of some positive embryo properties (Amemiya 1964; Niederwieser *et al*. 1990; Pecket and Selim 1965; Sharma *et al*. 1996). Based on the obtained, results from sucrose in the middle-level concentration of 45 g L^{-1} has increased development of embryos into plantlets and some growth parameters, similar results published by Valdez and Ulanovsky (1997); Wakana *et al*. (2003); Xu *et al*. (2005); Bharathy *et al*. (2005); Valdez (2005); Nookaraju *et al*. (2007); Tian *et al*. (2008); Tang *et al*. (2009) and Guo *et al*. (2011) has been well documented that middle concentration of sucrose can be used for better embryo germination and plantlet growth parameters. Furthermore, sugar and gibberellic acid concentration play a key role in embryo rescue success (Guo *et al*. 2004). Based on our observations, the increase of the GA_3 concentrations ensured higher germination percentages. In line with our results, Yamashita *et al*. (1998); Singh *et al*. (2011), and Ji *et al*. (2013), stated that the addition of GA_3 at high concentration (0.4, 0.5 mg L^{-1}) can enhance the development and growth of embryos. In a similar study, Guo *et al*. (2004) reported that the best result for grape embryo rescue occurred in the medium supplemented with high GA_3 concentration (GA_3, 0.5 mg L^{-1}), which is completely in agreement with our result. Our results are in contrast with the data of Burger and Goussard (1996) and Wakana *et al* (2003) who found that GA_3 has no significant effect on embryo rescue of Grape species.

Conclusions

In conclusion, the results of this study indicated that sugar is fundamental for embryo germination as well as GA_3 levels. The best explants, with a proportional shape between aerial part and root apparatus, were observed when the high level of sucrose and GA_3 were supplied; in this case, it was possible to observe many lateral roots and expanse leaves, useful parameters related to further acclimatization.

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