

# Plant regeneration from immature seeds of *Eugenia myrtifolia* Sims.

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**Abstract** *Eugenia myrtifolia* Sims. is an evergreen shrub, native to temperate and tropical rainforests of Australia, which is becoming an important containerized ornamental plant in the US and Mediterranean nursery industries. To satisfy the growing market demands for this new ornamental plant, development of an accelerated propagation method is required. The goal of this study was to investigate the *in vitro* regeneration potential of *E. myrtifolia* Sims. seeds at different stages of development, towards establishment of an *in vitro* multiplication system. Maximum regeneration of adventitious shoots was achieved from immature seeds cultured in the dark on half-strength Murashige and Skoog (MS) macronutrients and full-strength MS micronutrients and vitamins (MS/2) medium supplemented with 2.5  $\mu$ M thidiazuron (TDZ). Induction of regeneration occurred after at least two successive subcultures on TDZ-enriched medium, followed by subcultures on expression medium (hormone free MS/2) or multiplication medium [MM; MS medium enriched with 4.4 M 6-benzyladenine and 0.05 M  $\alpha$ -naphthaleneacetic acid], where complete development of shoots occurred. The regenerated shoots were excised and

transferred again onto MM for micropropagation, where a proliferation rate of 1:4 was achieved, and finally the shoots were transferred to a hormone-free MS medium for rooting. Following *ex vitro* transplanting, acclimatization over a period of 15 d was sufficient to establish greenhouse plants. The regenerated plants grown in the field for more than 2 yr showed the same phenotype as that of mother plants. The adventitious regeneration and micropropagation carried out in this study can be used for a large-scale propagation and genetic engineering of *E. myrtifolia* Sims.

**Keywords** *Eugenia myrtifolia* Sims. · *Syzygium paniculatum* Gaertn. · Ornamental plant · *In vitro* regeneration · Organogenesis · Immature seeds

## Introduction

*Eugenia myrtifolia* Sims. (syn. *Syzygium paniculatum* Gaertn., also called “brush cherry”, Fam. Myrtaceae) is an evergreen shrub, native to temperate and tropical rainforests of eastern Australia, which can reach up to 15 m height. Over the last few years, the plant has been promoted as a containerized ornamental plant in the nursery industry of US and in the Mediterranean countries of Europe, and is becoming an economically important product in ornamental trade worldwide. The popularity of *E. myrtifolia* as an ornamental plant comes from its dense, attractive foliage and from its relatively carefree management. During spring and summer, it produces clusters of white flowers, followed by red to dark red at maturity, edible fruits that develop over 2 mo. The fruits are cherry-sized, with crispy flesh surrounding a pea-sized seed (Fig. 1a).

This species is usually propagated by seeds, but cuttings are also frequently used, particularly for specific cultivars

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This paper is dedicated to the memory of Dr. Carmine Damiano, teacher and friend.

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**Figure 1.** *E. myrtifolia* Sims. (a) *In vivo* ripe fruits; (b) fruits at different stages of ripening; (c) fruit and seed at stage 3; (d–f) apomictic embryos developed by *E. myrtifolia* seeds on different media, attached or separated from the cotyledons; (g) seed on MS/2 medium enriched with 0.5  $\mu\text{M}$  2,4-D; (h) seed on MS/2 medium enriched with 0.5  $\mu\text{M}$  2,4-D and 0.5  $\mu\text{M}$  TDZ; (i) seed on MS/2 medium with 2.5  $\mu\text{M}$  TDZ; (j) and (k) regenerating callus developing from embryo axes of seed on 0.25  $\mu\text{M}$  (j) and 1  $\mu\text{M}$  (k) TDZ; (l) germinating seed on hormone-free MS medium. Bar=5mm



such as “Verlaine” or “Newport” in order to keep the true-to-type character (Lebrun et al. 1998).

*In vitro* propagation methods are powerful tools for germplasm conservation and mass production. *In vitro* culture techniques can also be used to induce genetic variability, namely somaclonal variation (Larkin and Scowcroft 1981), which can produce new genetic traits.

To date, *in vitro* culture of *E. myrtifolia* has not been reported. This study aimed to develop an efficient and reproducible protocol for regeneration of adventitious shoots and micropropagation of brush cherry. Leaf disks are the most common choice of explants in plant regeneration studies. However, our preliminary attempts to induce

adventitious shoots regeneration from leaf disks of brush cherry were not successful. Therefore, in the present study, immature seeds were used as starting explants. Murashige and Skoog (MS; Murashige and Skoog 1962) medium enriched with a synthetic cytokinin thidiazuron (TDZ) was applied to successfully induce adventitious shoot regeneration. Subsequently, optimal media for multiplication of these shoots and root formation were determined. The survival rate of transplanted *ex vitro* plantlets was above 90%. This study leads towards a defined and reliable micropropagation protocol for *E. myrtifolia*. This is the first report on *in vitro* culture and adventitious shoots regeneration of this ornamental species.

## Materials and Methods

**Plant material.** Fruits of *E. myrtifolia* Sims. were collected from September to October over three consecutive yrs 2009, 2010, and 2011, from an adult plant of “Tarantino Nursery” (Lecce, Italy). The fruits were collected from a single mother plant in order to minimize the effect of genetic variability.

The fruits were surface-sterilized by several steps: (1) extensive washing under running tap water, (2) submerging for 5 min in 80% (v/v) ethanol, (3) submerging for 20 min in 30% (v/v) commercial bleach, and (4) three or more rinses in sterile distilled water. Subsequently, the seeds were removed from the fruits using a blade and forceps. The external integuments were also removed. This step was easy to perform for immature seeds (stages 1–2) and for seeds from the “turning-pink” fruit (stage 3), when the integument was reddish (Fig. 1c). However, removal of integument was difficult to perform for seeds from ripe and over ripe fruits (stages 4 and 5, integument browning red), as it remained strongly attached to the seed.

**Reagents.** MS medium, agar (plant agar), and plant growth regulators TDZ, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), and  $\alpha$ -naphthaleneacetic acid (NAA) were purchased from Duchefa Biochemie (Haarlem, Netherlands). Ultrapure water from Milli-Q apparatus (Merk Millipore) was used to prepare culture media.

**Culture media and conditions.** The explants (seeds) were placed onto a regeneration medium (RM), in a sterile Petri dish (Corning Inc., 5.5 cm diameter), one explant per dish, and kept in the dark for 1 mo. The RM included half-strength MS macronutrients and full-strength MS micronutrients and vitamins (called in Duchefa catalog “modification no 1B”, here shortly MS/2 medium) enriched with growth regulators, as described below. The expression medium (EM) designed to elongate adventitious buds into shoots was a hormone-free MS/2. The multiplication medium (MM) consisted of MS medium enriched with 4.4  $\mu$ M BA and 0.05  $\mu$ M NAA, added before autoclaving. The pH of all media (RM, EM, and MM) were adjusted to 5.7 with 1 M KOH before the addition of 0.8% (w/v) agar. The media were autoclaved at 121°C for 20 min.

All *in vitro* cultures were maintained in a growth room at  $25 \pm 2^\circ\text{C}$ , in the dark or under a light intensity of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by Philips TLD/83 white fluorescent tubes, positioned 40 cm above the level of the cultures, with a 16-h photoperiod.

**Development of regeneration medium.** The effect of 2,4-D/TDZ factorial combination on the induction of regeneration process, as reported by Parra and Amo-Marco (1998)

was evaluated. A filter-sterilized auxin (2,4-D) and cytokinin (TDZ), applied individually and in combinations (at concentrations of 0, 0.5, and 2.5  $\mu$ M), were added to the autoclaved media (Table 1). Ten explants (mixed stages 3 and 4) per each of the nine media treatments were placed in the dark for a period of 90 d. Subcultures were carried out every 30 d. This experiment was performed to establish the optimal composition of the RM.

**Effect of seed development stage on regeneration.** The effect of seed development stage on regeneration capacity was evaluated. Seeds used as starting explants were picked at five different development stages (from immature to over-ripe; Fig. 1b) as follows: stage 1, 30 d after flowering (daf); stage 2, 40 daf; stage 3, 50 daf; stage 4, 60 daf; and stage 5, 70 daf. Ten explants representing each development stage were placed on regeneration medium RM7. The explants were placed in the dark and monitored over 90 d. Subcultures were carried out every 30 d.

**Effect of TDZ on regeneration from seeds at stage 3.** The objective of this experiment was to fine-tune the composition of RM for intensive regeneration of adventitious buds from the seeds at stage 3. TDZ applied individually was added to MS/2 medium at 0.25, 1.0, 2.5, 5.0, and 10  $\mu$ M. More than 20 fruits were used per medium treatment. The explants were placed in the dark, subcultured every 30 d and monitored over 90 d.

**Shoot elongation, micropropagation, and development of roots.** Explants with adventitious buds, induced on the surface of mother explants over three subsequent subcultures on RM medium, were placed on EM under the light for 2 wk to produce shoots. Subsequently, the elongated shoots were excised and transferred to MM under the light. For the establishment of MM composition, two levels of BA (4.4 and 6.6  $\mu$ M), each enriched with 0.05  $\mu$ M NAA, in MS medium were evaluated. The cultures were maintained in the light, subcultures were carried out every 30 d. Shoots from multiplication stage were also induced to root on hormone-free MS medium for 1 or 2 wk, until the roots were developed enough but not too much (plantlets were 4–5 cm tall, with roots 1–2 cm long).

**Table 1.** Combinations of growth regulators 2,4-D and TDZ (in micromolar) in regeneration media used for *E. myrtifolia* seed explants

2,4D/TDZ ( $\mu$ M)	0.0	0.5	2.5
0.0	RM1	RM4	RM7
0.5	RM2	RM5	RM8
2.5	RM3	RM6	RM9



**Acclimatization.** Rooted plants were gently washed in tap water to remove culture medium and were transplanted to small clay pots containing a mixture of sterilized soil and peat (1:1). Each pot was covered by a glass beaker to maintain high humidity for nearly 2 wk. Subsequently, the plantlets were gradually hardened in greenhouse over two more weeks, before transfer to open air.

**Statistical analysis.** Regeneration treatments were evaluated using 10–20 explants per treatment, arranged as a completely randomized design. Experiments were repeated twice. The data shown represent the mean  $\pm$  SD. Data were statistically analyzed using one-way analysis of variance using GraphPad Prism version 5.00 for Windows (Motulsky and Chrisopoulos 2003). Differences among means were evaluated using Tukey's test ( $P < 0.05$ ). Percentage values were subjected to arcsine transformation before analysis.

## Results

**Effect of growth regulators (2,4-D and TDZ) on induction of the regeneration process.** Isolated whole seeds of *E. myrtifolia* were placed on nine media treatments containing two growth regulators: 2,4-D and TDZ applied individually and in combination (Table 1). After 5 d of culture, development of a zygotic embryo shoot apex, and occasionally an apomictic embryo were observed (Fig. 1d–f). The embryos appeared randomly in all media tested, including the hormone-free medium (RM1; data not reported). This feature was not surprising since this species has been reported to be polyembryonic (Thurlby et al. 2011).

After just 15 d of culture, it became clear that 2,4-D applied individually (RM2 and RM3) induced only callusogenesis and rhizogenesis (Fig. 1g). The combination of both growth regulators, 2,4-D and TDZ (RM5, RM6, RM8, and RM9), induced rhizogenesis; however, apex proliferation was not observed (Fig. 1h). TDZ applied alone (RM4 and RM7) was identified as a potent inducer of multiple adventitious buds (Fig. 1i). After 30 d of culture on TDZ-enriched media (RM4 and RM7), development of a morphogenic callus with adventitious shoot primordia from embryo axes was observed (Fig. 1j and k). On medium lacking plant growth regulators (RM1), the seed gave rise to normal seedlings (Fig. 1l). The explants were subcultured two more times on their respective RM. Over the following 60 d of incubation, yet only the callus that was produced by explants from treatment RM7 induced shoot development. The regeneration efficiency achieved on this medium was 100%. Based on these observations, RM7 was selected as the optimal regeneration medium for *E. myrtifolia*.

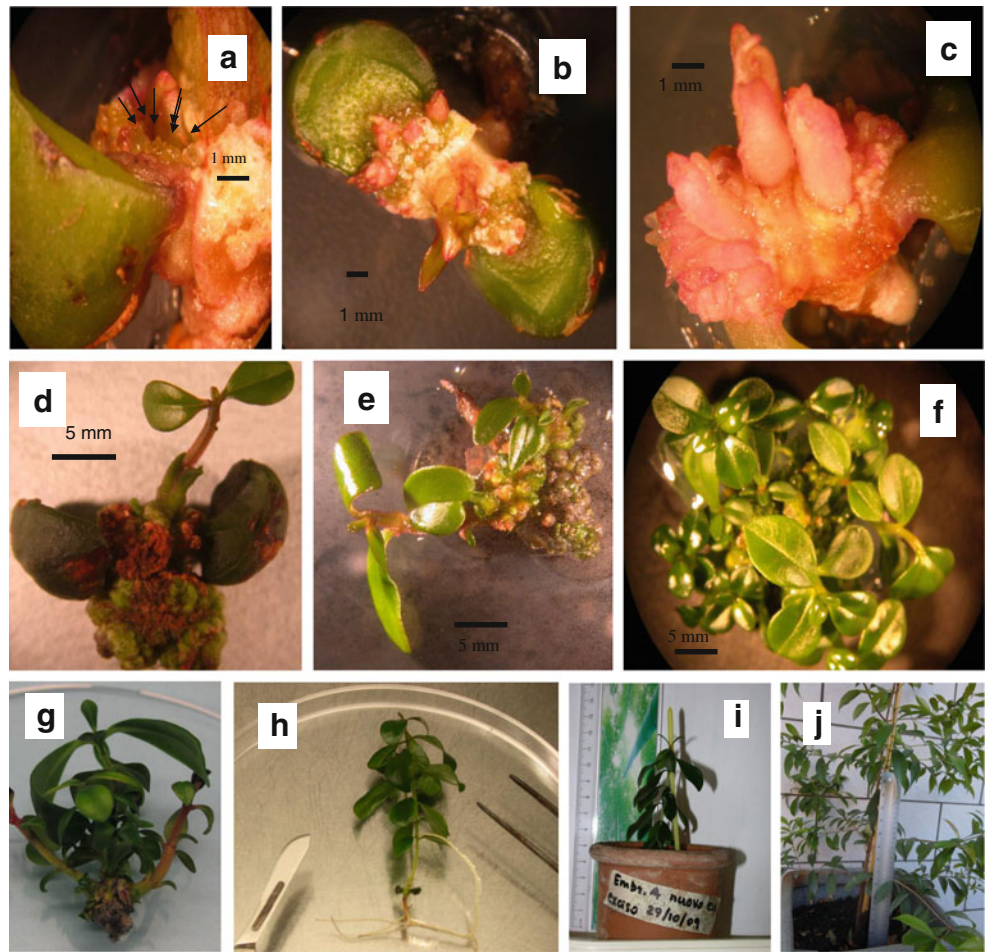
**Effect of seed development stage on regeneration capacity.** As the seed developmental stage affects the regeneration and morphogenic processes (Elhiti and Stasolla 2011), the effect of developmental stage on the regeneration of *E. myrtifolia* seeds was tested. The explants of seeds from five stages were incubated on RM7 medium, which effectively supported induction of adventitious buds in the former experiment. After 15 d of culture, it became clear that the efficiency of the regeneration process decreased with the seed maturation stage. The seeds representing stages 4 and 5 (ripe and over-ripe stages) remained inert. In contrast, the seeds representing all earlier stages of development (stages 1–3), where removal of the seed integument was possible, responded with active cell proliferation and subsequent formation of adventitious shoot primordia that were clearly visible after 15 d of culture (Fig. 2a). The most efficient formation of adventitious buds followed by vigorous growth of shoots was observed on stage 3 explants (Fig. 2b and c), and these were selected for further study.

The regenerated shoots were excised, transferred to EM, and placed under light for further development. At least two monthly subcultures on RM7 in the dark were required to facilitate a sufficient development of adventitious shoots that could successfully survive transfer to EM. If the regenerating cotyledons were transferred to EM earlier (i.e., after 30 d of incubation on RM), the regenerating young structures failed to develop further, became brown and necrotic (Fig. 2d).

**Effect of TDZ concentration on regeneration efficiency of stage 3 seeds.** The objective of this experiment was to understand the effect of various concentrations (from 0.25 to 10  $\mu$ M) of TDZ applied individually on the regeneration capacity of stage 3 seed in order to maximize the production of *E. myrtifolia* plantlets. After 30 d of incubation, regeneration of adventitious buds was observed in all media tested, with a maximum percentage of regenerating explants occurring on medium containing 2.5  $\mu$ M TDZ (Fig. 3). This medium was identical to the earlier selected RM7. At 90 d after induction, a regeneration rate of nearly 100% was observed for all media, with the exception of medium containing the lowest (0.25  $\mu$ M) dose of TDZ (Fig. 3). Moreover, explants cultured on the highest growth regulator levels became necrotic, indicating that high concentrations of TDZ were toxic to *E. myrtifolia* explants.

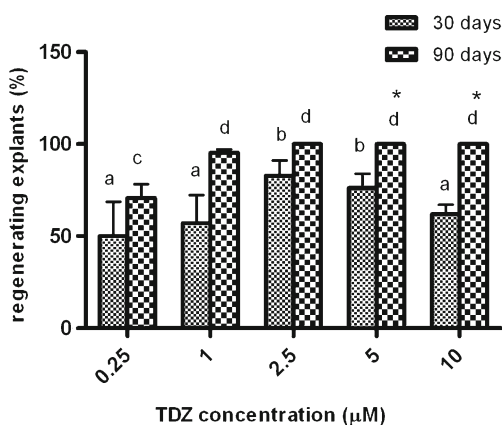
The mean number of regenerated structures per explant revealed through microscopic observation carried out after 90 d of culture on RM, indicated that media enriched with 0.25–2.5  $\mu$ M TDZ produced equally high numbers of shoots per explant (Fig. 4). These results confirmed that TDZ present in RM at 2.5  $\mu$ M facilitates an intensive regeneration of viable adventitious buds from stage 3 *E. myrtifolia* seed explants.

**Figure 2.** Regeneration process in *E. myrtifolia* immature seeds. (a) Adventitious shoot primordia on immature seed cultured for 15 d on RM7; (b) and (c) adventitious shoots regenerated from immature seed (stage 3) on RM7; (d) regenerating callus transferred under light after 30 d of incubation in the dark; (e) regenerating explants on EM; (f) regenerating explants on MM; (g) proliferating plantlets on MM medium; (h) *in vitro* rooted plantlets; (i) *in vivo* acclimatized plants; (j) 2-yr-old regenerated plants in the field.

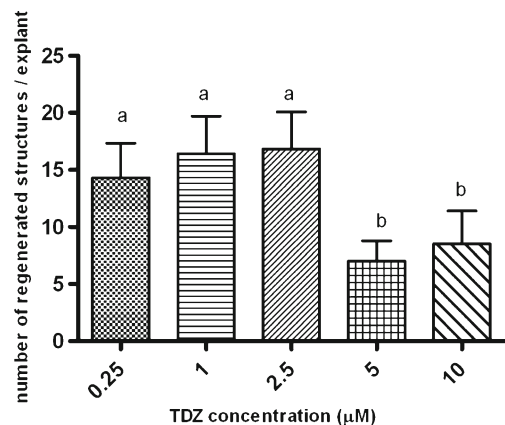


**Shoot elongation.** After 90 d of incubation on RM7, the explants (seed cotyledons with regenerated buds) were transferred to two different media, EM or MM, to induce shoot growth. Hormone-free medium is usually applied as

basic strategy for elongation of regenerated adventitious shoots and this approach has been applied in our study (EM). Although the number of adventitious buds identified on each explant varied from 12 to 20 (Fig. 4), after transfer



**Figure 3.** Percentage of regenerating *E. myrtifolia* cotyledons after 30 and 90 d on media supplemented with TDZ incubated in darkness. Asterisk Explants that had regenerated and become partially necrotic. Bars represent the means±SD. Data with the same letter in a column were not significantly different according to Tukey's multiple comparison test ( $P<0.05$ ).



**Figure 4.** Mean number of regenerated structures (shoots and shoot primordia) per explant from *E. myrtifolia* cotyledons cultured on media supplemented with TDZ, recorded after 90 d of culture in darkness. Bars means±SD. Data with the same letter in a column were not significantly different according to Tukey's multiple comparison test ( $P<0.05$ ).

to EM most of the regenerated structures failed to develop further and only two or three shoots were obtained per explant (Fig. 2e). On the contrary, after the transfer of explants to MM (a medium enriched with BA and NAA), on average  $22.6 \pm 5.5$  well-developed shoots were obtained per explant (Fig. 2f).

Beside the explants incubated on RM7, we have also attempted to regenerate shoots from explants incubated on media containing lower (0.25 and 1  $\mu\text{M}$ ) and higher (5 and 10  $\mu\text{M}$ ) levels of TDZ. The explants incubated on RM media containing lower levels of TDZ produced fewer plantlets. The explants incubated on RM media containing higher levels of TDZ produced distorted plantlets. These observations indicate that the choice of suitable growth regulators and their levels in the regeneration medium is a prerequisite of the subsequent successful development of shoots into plantlets.

#### *Micropropagation, development of roots, and acclimatization.*

Fully developed shoots excised from the regenerating explants can be used as secondary explants for multiplication (micropropagation) of the regenerated shoots. In the following experiment, excised shoots were placed on MS basal medium enriched with BA (4.4 or 6.6  $\mu\text{M}$ ) and 0.05  $\mu\text{M}$  NAA. With an increase of BA level in the culture medium, the proliferation rate (development of axillary buds into shoots) also increased; however, at higher levels of BA vitrification of shoots occurred. A satisfactory proliferation rate (1:4) and development of vigorous shoots (Fig. 2g) was achieved on medium containing 4.4  $\mu\text{M}$  BA and 0.05  $\mu\text{M}$  NAA, and this medium was selected as the MM for further studies. *E. myrtifolia* is a species that grows slowly in culture, and subsequently a 40-d subculture period was required for micropropagation.

Fully developed shoots of *E. myrtifolia* obtained after shoot multiplication steps, were placed on hormone-free MS medium for 2 wk to induce roots (Fig. 2h). Subsequently plantlets were transplanted into small clay pots for acclimatization (Fig. 2i).

In order to follow the phenotypic variation of regenerated plants, progeny from seven randomly selected regenerating seeds were carefully monitored for phenotypic variation. Progeny of each regenerating seed gave rise to several "lines", with each line representing an independent regeneration event. From the initial seven seeds selected, a total of 33 lines were regenerated and micropropagated, and monitored for phenotypic variation over a period of 2 yr. This included observations first in micropropagation conditions, then in glasshouse, and in the open field (data not shown). More than 90% of plantlets successfully acclimatized to field conditions and survived. The phenotype of all regenerated plants was uniform and true-to-type, as assessed over the 2-yr period (Fig. 2l).

*In vitro propagation scheme of E. myrtifolia.* The entire regeneration process of *E. myrtifolia* developed in this study can serve to develop the first micropropagation protocol for brush cherry. The most important points of this protocol are: (1) selection of immature seeds (stage 3) as they represent the most suitable explant material; (2) modified basal MS medium enriched with 2.5  $\mu\text{M}$  TDZ (RM7); (3) initiation of adventitious buds on RM7 from immature seeds required at least two monthly subcultures in the dark (giving a total regeneration period of 90 d); (4) adventitious buds form shoots on EM when kept under the light for 2 wk; and (5) adventitious shoots represent suitable secondary explants for shoot multiplication when transferred to MM. A scheme of the micropropagation protocol of *E. myrtifolia* is presented in Fig. 5.

## Discussion

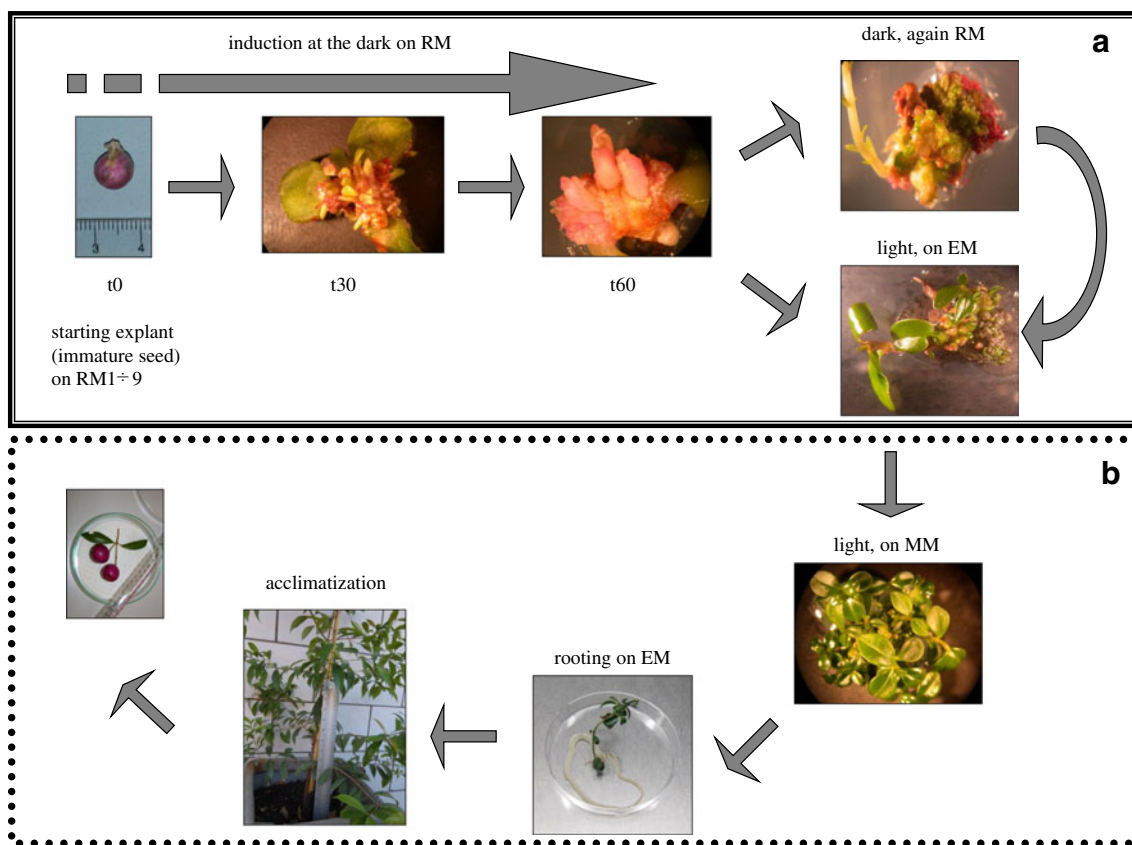
*E. myrtifolia* Sims. is an Australian ornamental species, with a growing interest in temperate climate nursery production. Beside propagation by traditional cuttings, *in vitro* techniques can be applied as an alternative method of accelerated vegetative propagation of this plant. Adventitious shoot regeneration could be used with the purpose for vegetative propagation or to induce genetic variability through somaclonal variation. In this study, the potential of *E. myrtifolia* Sims. immature seeds to regenerate *in vitro* adventitious shoots has been assessed for the first time.

TDZ is a synthetic cytokinin that is a potent inductor and regulator of plant morphogenesis, characterized by a unique mode of action, as the modulation of endogenous levels of growth regulators (Murthy et al. 1998). This growth regulator has been reported to induce organogenesis *in vitro* (Jones et al. 2007). Moreover, it has been widely used to induce regeneration in recalcitrant woody species because of its tremendous ability to stimulate shoot proliferation (both axillary and adventitious) in trees, characterized by a natural strong monopodial growth habits (Huetteman and Preece 1993).

Generally, stimulation of multiple shoot or bud formation is achieved by culturing explants on medium supplemented with relatively high levels of cytokinins. TDZ has been shown to promote differentiation of organized centers of growth in cultured tissues at much lower concentrations, and shoot regeneration occurs with an efficiency comparable to (or as greater than) that of other cytokinins (Murthy et al. 1998).

Due to its capability to satisfy both the auxin and cytokinin requirements, TDZ has been reported to induce *in vitro* regeneration also by somatic embryogenesis. In *Myrtus communis* L. (Fam. Myrtaceae), regeneration by somatic embryogenesis has been obtained from immature seeds on media supplemented with both, 2,4-D and TDZ (Parra and Amo-Marco 1998). Following the findings of Parra and





**Figure 5.** *E. myrtifolia* Sims. organogenesis from immature seed (a) and micropropagation of regenerated adventitious shoots (b). *RM* regeneration medium, *EM* expression medium, *MM* multiplication medium.

Amo-Marco (1998), the same hormonal combination was tested in this study. In addition, the mineral composition of half-strength MS macronutrients, also reported by Parra and Amo-Marco (1998), could be useful to induce the early morphogenic response (15 d). A medium poor in macronutrients is considered suitable for the development of the shoot apex. In fact, successful *in vitro* initiation of cultures from axillary buds requires very poor nutrients, particularly macrosalts (George and Sherrington 1984), and this approach was implemented in the development of the regeneration medium of *E. myrtifolia* in the present study. In contrast to *M. communis*, which under these medium conditions gave rise to somatic embryos (Parra and Amo-Marco 1998), *E. myrtifolia* immature seeds regenerated a high number of adventitious shoots. This result indicates that species belonging to the same family under the same conditions may exhibit various *in vitro* morphogenic responses.

In this study, immature seeds were chosen as the initial explants. In case of woody species, seed explants are more responsive to regenerate *in vitro*, particularly when TDZ is used. However, a disadvantage of seed use is the potential genetic variability, particularly in highly heterozygous woody species. We have evaluated the effect of five stages

of seed development on the regeneration process. Interestingly, all the explants that produced adventitious buds had the seed integuments removed. The integument represents a protective layer that surrounds an ovule and later becomes the seed coat. It is highly possible that the removal of the integument allowed a direct contact of the explant's tissue with medium components, which enhanced the accessibility of nutrients and growth regulators from the medium and subsequently contributed towards higher regeneration capacity of explants.

With regards to phenotypic traits, *E. myrtifolia* is a nonsegregating plant since it can be propagated by seeds giving apparent true-to-type progeny. For this reason we have considered seed as suitable explant source for the development of an *in vitro* regeneration system.

Adventitious regeneration can be used to vegetatively propagate a genotype, without inducing genetic variability. This is the case of *E. myrtifolia*, since nearly a hundred plants were generated in this study and variations at phenotypic level were not observed. *E. myrtifolia* is a polyembryonic species, as demonstrated by the development of nucellar embryos in some cases. This feature represents a genetic tendency to produce adventitious true-to-type regenerated plants. Therefore, the reliable regeneration

system defined in this study could be usefully applied for mass *in vitro* propagation or for genetic manipulation of this species.

## Conclusions

This is the first study aimed at the establishment of a micropropagation protocol for *E. myrtifolia*. Immature seeds collected 50 d after flowering, with integuments removed, were identified as the most suitable starting explant source. Modified basal MS medium enriched with 2.5  $\mu\text{M}$  TDZ was identified as the most efficient medium for regeneration of adventitious buds from seed cotyledons. Adventitious buds successfully developed into shoots when transferred onto hormone-free MS/2 medium under light over 2 wk. Isolated shoots were proliferated on 4.4  $\mu\text{M}$  BA and 0.05  $\mu\text{M}$  NAA-enriched MS medium and rooted on hormone-free MS medium over subsequent 2 wk. The complete micropropagation cycle developed in this study requires approximately 4 mo and produces about five plants per explant. The plantlets survival rate in the field is 90%. The progeny monitored over 2 yr was true-to-type.

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