# *In vitro* establishment and micropropagation of mango (*Mangifera indica* L.) from cotyledonary nodes

**Francisco Conde[1] · Elizabeth Carmona-Martin[1] · Jose I. Hormaza[1] · Cesar Petri[1]**

## Abstract

Mango is the fifth most important fruit crop in the world in terms of production with an increasing demand for high-quality plant material for new plantings. Compared to other fruit tree crops, vegetative propagation in mango is slow and allows only a relatively limited production of plant material. To date, efficient procedures for *in vitro* establishment and micropropagation are not yet available in mango. This work aims at filling this gap. Germination of mango seeds *in vitro*, compared with *ex vitro* conditions, significantly increased the germination rate for the monoembryonic genotype tested ('Irwin'). *In vitro* germination also increased the number of developed embryos for the two polyembryonic genotypes analysed, 'Ataulfo' and 'Gomera-4'. Regarding the use of shoot tips for *in vitro* establishment and micropropagation, our results confirmed that this explant is not adequate for this purpose. We report for the first time the use of cotyledonary nodes as initial explants in mango. Axillary shoots were obtained in all the genotypes tested ('Ataulfo', 'Sabre', 'Gomera-4', 'Irwin' and 'Keitt'), although the regeneration rate was highly genotype-dependent. Thidiazuron induced high-frequency regeneration patterns. The best results were observed with 'Keitt'. When 3.0 mg l$^{-1}$ thidiazuron was added to the medium, a 63.15% regeneration rate was reached and about 4 shoots per regenerating explant were obtained. Subsequently, microshoots excised from the cotyledonary nodes were successfully rooted *in vitro* and acclimatized to *ex vitro* conditions. Our results show that the use of cotyledonary nodes is efficient for mango mass propagation and, consequently, represents a qualitative advance for *in vitro* propagation of this recalcitrant species.

**Keywords** Anacardiaceae · Germination · Organogenesis · Shoot multiplication · Shoot regeneration · Fruit crops

## Introduction

Mango (*Mangifera indica* L.), a member of the Anacardiaceae, is ranked fifth in production among fruit crops worldwide after *Musa* sp. (bananas and plantains), *Vitis* sp. (grape), *Citrus* sp. and *Malus* sp. (apple) (FAOSTAT 2021). The continuous expansion of global mango production is resulting in an increasing demand for high-quality plant material usually grafted on seedling rootstocks. There are two different groups of mangoes distinguished by their mode of reproduction and origin: the subtropical group or Indian type, with monoembryonic seeds, and the tropical group or Southeast Asian type, with polyembryonic seeds (Mukherjee and Litz 2009). Polyembryonic mangoes have one zygotic embryo and a variable number of nucellar embryos, depending on the genotype. Polyembryonic mangoes are frequently used as rootstocks since the nucellar embryos allow clonal propagation. However, mango grafting is a slow process that allows only a relatively limited production of trees.

Micropropagation would allow massive plant production for nurseries and growers. Nevertheless, mango is a very recalcitrant species for *in vitro* culture, mainly due to high phenolic exudation, media browning, explant necrosis, endogenous contamination and/or slow growth response (Petri *et al*. 2021). To date, efficient procedures for *in vitro* establishment and micropropagation are not yet available in mango.

Attempts to establish *in vitro* mango clonal material have failed with different types of explants such as nodal explants,

Francisco Conde and Elizabeth Carmona-Martin contributed equally to this work.

✉ Cesar Petri
  cesar.petri@csic.es

[1] Departamento de Fruticultura Subtropical Y Mediterránea, Instituto de Hortofruticultura Subtropical Y Mediterránea, UMA-CSIC, Avenida Dr. Wienberg, S/N. 29750, Algarrobo-Costa, Málaga, Spain

shoot tips and stem nodes (Yang and Lüdders 1993; Thomas and Ravindra 1997; Chandra *et al.* 2004; Samaan *et al.* 2007; Krishna *et al.* 2008). As a tool to increase the recovery rate of hybrids for subsequent evaluation in mango breeding programs, *in vitro* culture of immature embryos has also been explored (Dinesh *et al.* 2005; Pérez-Hernández and Grajal-Martín 2011; Petri *et al.* 2021). In some cases, complete plantlets were obtained at a frequency of around 83% (Pérez-Hernández and Grajal-Martín 2011).

The objective of this work was to develop procedures for *in vitro* establishment and micropropagation of mango juvenile material from mature seed-derived tissues towards the optimization of a reliable micropropagation procedure. Although in monoembryonic genotypes true-to-type lines would not be produced, juvenile material could aid to establish reliable procedures (media, culture conditions *etc.*) to be applied with mature/clonal tissues in future experiments. To the best of our knowledge, there is no information about *in vitro* germination of mature zygotic or nucellar mango embryos.

The utilization of cotyledonary node explants for the micropropagation of mango has not been reported yet. This explant consists of the section of the embryo axis (of about 1 cm in length) where the cotyledons are inserted into the axis. The removal of the apical dominance, by cutting out the main shoot, together with the application of exogenous plant growth regulators promotes the development of new axillary shoots. Cotyledonary nodes have been used successfully for the micropropagation of *Anacardium occidentale* (Nanti *et al.* 2020), another member of the Anacardiaceae, and other woody species such as *Cassia sophera* (Parveen and Shahzad 2010), *Cajanus cajan* (Sharma *et al.* 2006) or *Sterculia urens* (Hussain *et al.* 2007). In this work, we show, for the first time, the achievement of shoot regeneration from mango cotyledonary nodes. Thereafter, the microshoots were successfully rooted *in vitro* and acclimatized to *ex vitro* conditions.

Taking into account the high recalcitrance of mango to *in vitro* culture and the lack of current efficient propagation procedures, these results represent a step forward towards the obtention of efficient micropropagation protocols in this species.

## Materials and methods

**Plant material** Mature fruits, approximately 4 mo after full bloom, from monoembryonic ('Irwin' and 'Keitt') and polyembryonic ('Ataulfo', 'Sabre' and 'Gomera-4') mango genotypes, grafted on 'Gomera-4' rootstocks, were harvested from the germplasm collection located at the IHSM-UMA-CSIC "La Mayora" (Algarrobo-Costa, Malaga, Spain).

**In Vitro Seed Germination** For seed disinfection, entire fruits were washed with a soap solution and tap water and the seeds with the endocarp were extracted from the mesocarp of the fruit. Complete seeds, with the endocarp, were immersed in a solution of 30% (v/v) commercial bleach and 0.02% (v/v) Tween-20 for 30 min and then rinsed three times with sterile distilled water under a laminar air flow hood. Sterilized pruning shears were used to open the endocarp. The seed coat was removed with the aid of a scalpel (Fig. 1a). Seeds were placed on a germination medium (GM) (Souza *et al.* 2020), consisting of half-strength MS (Murashige and Skoog 1962) salts, 30 g l$^{-1}$ sucrose, 0.5 mg l$^{-1}$ gibberellic acid (GA), 100 mg l$^{-1}$ cysteine and 0.7% agar (Micropropagation Type I, Caisson Labs, Smithfield, UT). Cultures were maintained for 3 wk at $25 \pm 1$ °C in darkness. Germination rate, number of shoots in polyembryonic genotypes, length of the shoots and contamination rates were recorded 3 wk after culture initiation.

**Ex Vitro Seed Germination** Pruning shears were used to open the endocarp and extract the seeds. Seeds were planted in pots containing 60% coconut fibre, 30% commercial substrate (Turbas y Coco Mar Menor S.L., Spain) and 10% litonite and cultured in a greenhouse in darkness. Germination rates, number of shoots and length of the shoots were recorded after 3 wk since the culture initiation.

**In Vitro Establishment of Shoot Tips** From *in vitro* material, 3–5-cm-long apical shoots were obtained from *in vitro* germinated seeds 3 wk after germination (genotypes 'Irwin' and 'Ataulfo') and placed on two culture media, which only differed in the plant growth regulators added. Culture media were based on the medium described by Chandra *et al.* (2004). Briefly, the media consisted of MS salts and vitamins, 45 g l$^{-1}$ sucrose, 100 mg l$^{-1}$ ascorbic acid, 100 mg l$^{-1}$ polyvinylpyrrolidone, 100 mg l$^{-1}$ casein hydrolysate and 7 g l$^{-1}$ of agar (Micropropagation Type I, Caissonlab). We added to this recipe 100 mg l$^{-1}$ lipoic acid. Medium 1 (MI1) and medium 2 (MI2) were supplemented with 1 mg l$^{-1}$ 6-benzylaminopurine (BAP) and 1 mg l$^{-1}$ zeatin or with 1 mg l$^{-1}$ indole acetic acid (IAA) and 3 mg l$^{-1}$ kinetin, respectively. The pH was adjusted to 5.7 before autoclaving for 20 min at 121 °C and 1.05 kg cm$^{-2}$. Cultures were incubated at $25 \pm 1$ °C with a 16 h day photoperiod under cool-white fluorescent tubes (F40 tubes Gro-lux, Sylvania España, Madrid, Spain) with 45 μmol m$^{-2}$ s$^{-1}$ light intensity (wavelength 400–700 nm).

Likewise, about 3–5-cm-long apical shoots (genotypes 'Irwin' and 'Ataulfo'), obtained from *ex vitro* germinated seeds 3 wk after germination, were disinfected by immersion in a 1% (v/v) sodium hypochlorite solution with 0.02% (v/v) Tween-20 for 20 min. Then, explants were rinsed three times with sterile distilled water in aseptic conditions, placed onto MI1 or MI2 medium and cultured under the conditions described above.

**Figure 1.** (*a*) Removal of the seed coat with a scalpel in a laminar flow hood. *Horizontal bar* indicates 1 cm. (*b*) Cotyledonary node explant after preparation. *Horizontal bar* indicates 1 cm. (*c*) *Ex vitro* germinated seeds of cv. 'Irwin' (monoembryonic) after 3 wk of culture. *Horizontal bar* indicates 5 cm. (*d*) *In vitro* germinated seed cv. 'Ataulfo' (polyembryonic) after 3 wk of culture. *Horizontal bar* indicates 5 cm. (*e*) Necrotic apical shoot (cv. 'Irwin') after 4 wk of culture. Medium browning is apparent. *Horizontal bar* indicates 1 cm. (*f*) Apical shoot tip (cv. 'Ataulfo') 5 mo after *in vitro* establishment. *Horizontal bar* indicates 1 cm. (*g*, *h*) Cotyledonary nodes showing regeneration after 8 wk of culture. *Horizontal bar* indicates 1 cm. (*i*) Multiple shoot regeneration from a cotyledonary node cultured onto MI2 medium with 3.0 mg l$^{-1}$ TDZ. *Horizontal bar* indicates 1 cm. (*j*) Cotyledonary node of cv. 'Keitt' 3 wk after *in vitro* seed germination. Axillary shoot already developed (*black arrow*). *Horizontal bar* indicates 1 cm. (*k*) Cotyledonary node of cv. 'Keitt' after 8 wk of culture. Previously developed shoot (*black arrow*) and new axillary shoot (*white arrow*) visible. *Horizontal bar* indicates 1 cm.
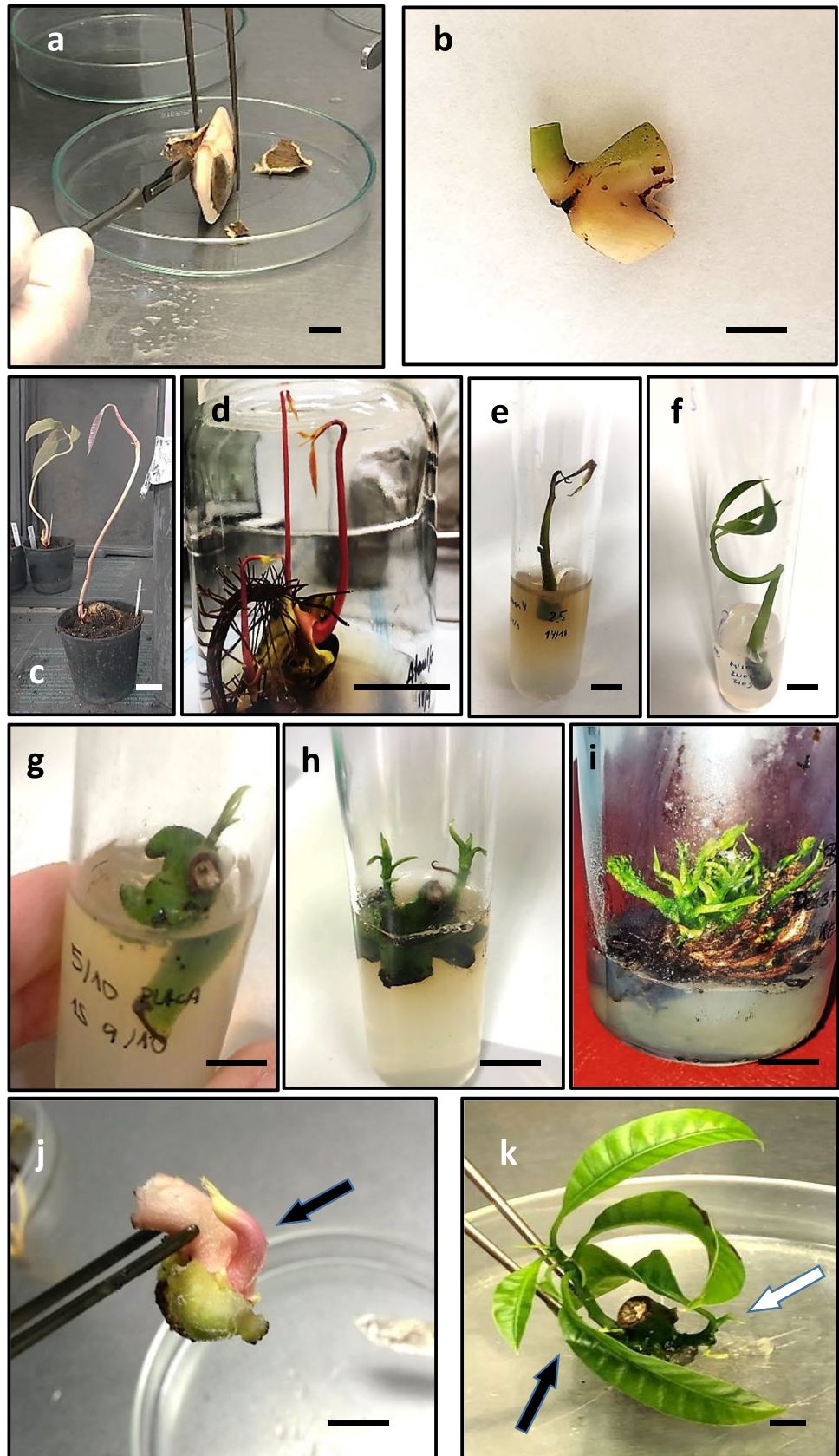
In both *in vitro* and *ex vitro* shoot tips, the rates of contamination, necrosis and survival were recorded 4 wk after the beginning of the experiment.

### *In Vitro* Establishment of Cotyledonary Node Explants

Cotyledonary nodes of 'Ataulfo', 'Irwin', 'Gomera-4' and 'Sabre' were collected from *in vitro* germinated seeds in GM medium 3 wk after germination. For explant preparation, cotyledons, shoots and roots were removed using a scalpel, leaving approximately 1 cm of each tissue (Fig. 1*b*). Cotyledonary nodes were placed on MI2 and cultured at $25 \pm 1$ °C with a 16 h day photoperiod under cool-white fluorescent tubes (F40 tubes Gro-lux, Sylvania España, Madrid, Spain) with 45 µmol m$^{-2}$ s$^{-1}$ light intensity (wavelength 400–700 nm).

To study the effect of GA on cotyledonary node regeneration, seeds of 'Keitt' were germinated *in vitro* on GM medium with the addition of different GA concentrations (0.0, 0.5 or 1.0 mg l$^{-1}$). After 3 wk of culture on GM, the germination rates, number of cotyledonary nodes that already showed axillary shoots, number of shoots per regenerating explant and length of the axillary shoots were recorded. Then, cotyledonary nodes were collected and cultured on an MI2 medium (containing 1.0 mg l$^{-1}$ IAA and 3.0 mg l$^{-1}$ kinetin) with the conditions described above. After five additional weeks on MI2, regeneration rates, the number of shoots per regenerating explant and the length of such shoots were recorded.

To study the effect of thidiazuron (TDZ) on the regeneration from the cotyledonary nodes, seeds of 'Keitt' were germinated *in vitro* on GM medium without GA. After 3 wk, explants were collected and cultured on MI2 medium following 4 different cytokinin treatments; 3.0 mg l$^{-1}$ kinetin (the original recipe above), or kinetin substituted with 1.0, 2.0 or 3.0 mg l$^{-1}$ TDZ. Five weeks later, regeneration rates, the number of shoots per regenerating explant and the length of such shoots were recorded.

### Rooting and Acclimatization

When the shoots generated from the cotyledonary nodes reached 1.5–2.0 cm long, they were excised from the explant and rooted following a two-step protocol previously described for mango microshoots (Ara *et al*. 1998).

For acclimatization, rooted shoots were washed in water to eliminate agar residues and transferred to pots containing 60% coconut fibre, 30% commercial substrate (Turbas y Coco Mar Menor S.L., Spain) and 10% litonite. The potted plantlets were placed into plastic bags that were sealed and maintained in a growth chamber with a 16/8 h light/dark cycle, light intensity of 45–50 µmol m$^{-2}$ s$^{-1}$, 60% relative humidity and a temperature of $26 \pm 1$ °C. The plastic bags were opened gradually and, after 2 wk, they were fully opened. After 1 wk in opened plastic bags, with irrigation applied as needed, plastic bags were finally removed and plants were transferred to a greenhouse.

### Microsatellites (Short Sequence Repeats; SSRs) Analyses

DNA of 8-wk-old shoots germinated from polyembryonic seeds was extracted following the protocol described by Viruel and Hormaza (2004). A total of 9 highly polymorphic mango SSR loci (Lmma1, Lmma4, Lmma6, Lmma7, Lmma8, Lmma9, Lmma10, Lmma11, Lmma15) were analysed as described previously (Viruel *et al*. 2005).

### Statistical Analyses

Categorical data (percentages) were compared by Fisher's exact test. Continuous variables (stem length, number of shoots per explant *etc*.) were transformed when needed to fit with a normal distribution and analysed by ANOVA. Student's *t*-test was applied to separate means.

## Results

### *In Vitro vs. Ex Vitro* Germination

Seed germination, *in vitro* and *ex vitro*, was clearly visible after 3 wk of culture (Fig. 1*c, b*) and data were collected (Table 1).

The efficiency of the seed disinfection procedure applied before *in vitro* germination varied depending on the genotype, resulting in a low ratio of contaminated seeds for 'Irwin' (5.4%), intermediate for 'Ataulfo' (29.0%),

**Table 1.** *In vitro* and *ex vitro* seed germination of the mango genotypes tested

| Genotype | Number of seeds | | *In vitro* contamination ($\% \pm$ S.E.) | Germination ($\% \pm$ S.E.) | |
|---|---|---|---|---|---|
| | *In vitro* | *Ex vitro* | | *In vitro* | *Ex vitro* |
| 'Irwin' | 37 | 28 | $5.4 \pm 3.7$ | $94.6 \pm 3.7$ a | $71.4 \pm 7.4$ b |
| 'Ataulfo' | 31 | 22 | $29.0 \pm 8.1$ | $93.5 \pm 4.4$ a | $95.4 \pm 3.7$ a |
| 'Gomera-4' | 24 | 24 | $25.0 \pm 8.8$ | $95.8 \pm 4.1$ a | $91.7 \pm 5.6$ a |
| 'Keitt' | 75 | n/a | $25.3 \pm 5.0$ | $86.7 \pm 3.9$ | n/a |
| 'Sabre' | 25 | n/a | $72.0 \pm 9.0$ | $100 \pm 0.0$ | n/a |

Different letters within the same row indicate significant differences between germination treatments ($p \leq 0.05$). *n/a* not assayed
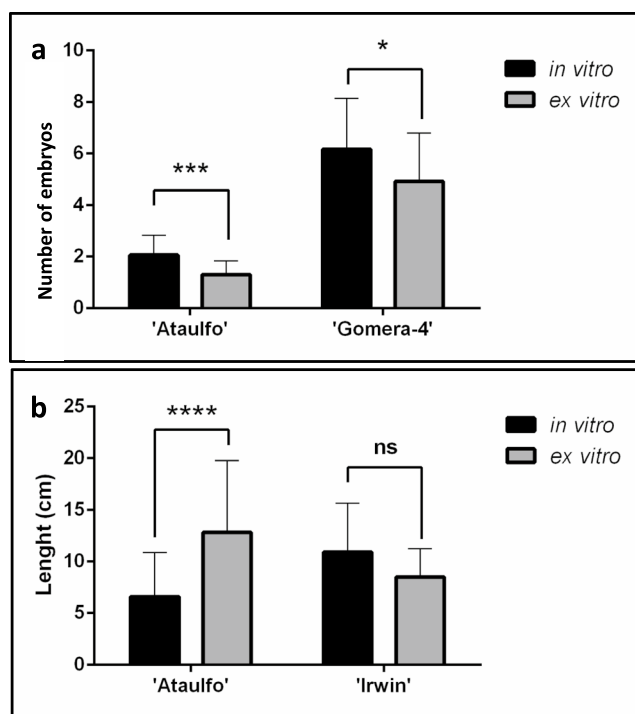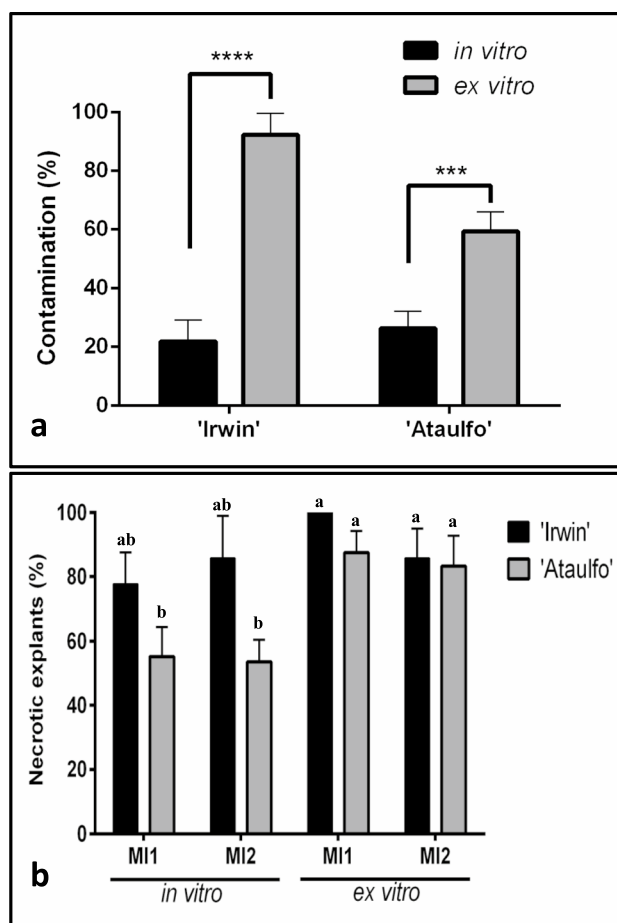
**Figure 2.** Mango seed germination. (*a*) Number of germinated embryos per seed in polyembryonic mangoes after 3 wk of culture. (*b*) Length of the shoots after 3 wk of culture. *Asterisks* indicate significant differences between treatments; ns, not statistically significant; *$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$. A total of 37, 31 and 24 mature seeds were used in this study for 'Irwin', 'Ataulfo' and 'Gomera-4', respectively.



**Figure 3.** Mango shoot tips. (*a*) Contamination rates (%) of shoot tip explants after 4 wk of culture. *Asterisks* indicate significant differences between treatments, ***$p \leq 0.001$, ****$p \leq 0.0001$. (*b*) Explant necrosis rates (%) after 4 wk of culture. Different *letters* indicate significant differences ($p \leq 0.001$). A total of 39 and 67 explants were used in this study for 'Irwin' and 'Ataulfo', respectively

'Gomera-4' (25.0%) and 'Keitt' (25.3%) and high for 'Sabre' (72.0%).

'Irwin' germination percentage was significantly higher ($p \leq 0.05$) *in vitro* than *ex vitro* (Table 1). Germination rates for 'Ataulfo' and 'Gomera-4', the two polyembryonic genotypes studied, were similar *in vitro* and *ex vitro* (Table 1). However, the number of germinated embryos per seed at 3 wk showed a significant increase ($p \leq 0.001$ for 'Ataulfo' and $p \leq 0.05$ for 'Gomera-4') *in vitro* compared with *ex vitro* conditions (Fig. 2a). On an average, 'Ataulfo' developed 1.3 embryos *ex vitro* and 2.1 *in vitro*, and 'Gomera-4' 4.9 *ex vitro* versus 6.2 *in vitro*.

The length of the shoots was also affected by the germination conditions (Fig. 2b). For 'Ataulfo' (polyembryonic genotype), shoots were significantly longer ($p \leq 0.0001$) under *ex vitro* conditions (12.8 cm on average) compared with *in vitro* (6.6 cm on average). In contrast, for the monoembryonic genotype 'Irwin', the length of the shoots was similar in both treatments.

***Apical Shoot Establishment*** Apical shoots of 3–5 cm long from 'Ataulfo' and 'Irwin' seedlings, germinated *in vitro* or *ex vitro*, were collected and cultured on two different media:

MI1, with the cytokinins BAP and zeatin, and medium MI2 containing the cytokinin kinetin and the auxin IAA.

Shoot tips from *ex vitro* seedlings were decontaminated as described in "Materials and Methods". For shoot tips derived from *in vitro* germination, aseptic manipulation in a laminar flow cabinet was considered enough. After 4 wk of culture, we evaluated the degree of contamination. Microbial contamination was significantly lower ($p \leq 0.0001$ and $p \leq 0.001$ for 'Irwin' and 'Ataulfo', respectively) in explants from *in vitro* germinated seeds compared with those from *ex vitro* seeds (Fig. 3a). Around 22.0% of 'Irwin' shoot tips from *in vitro* germinated seeds were contaminated compared to 92.3% of the shoots from *ex vitro* germinated seeds. Similar results were obtained for 'Ataulfo', where 26.0% of the shoot tips from *in vitro* germinated seeds were contaminated, versus 59.3% contaminated shoot tips from *ex vitro* germinated seeds (Fig. 3a).

We also evaluated the survival of the shoot tips after 4 wk of culture. At least half of the explants were necrotic for both genotypes tested, regardless of the germination conditions or of the medium where shoot tips were placed (Figs. 1*e*, 3*b*). The genotype and the explant origin affected the explant necrosis rate. In 'Ataulfo', the necrosis rate was significantly reduced ($p \leq 0.001$) in explants from *in vitro* germinated seeds compared with those explants from *ex vitro* germinated seeds. Conversely, no differences were observed in 'Irwin' (Fig. 3*b*). Within the same genotype, no differences in explant necrosis rate were observed between the media used for shoot establishment never affected (Fig. 3*b*). Regardless of the medium used, overall explant necrosis rates were 81% and 92% for 'Irwin' and 54% and 85% for 'Ataulfo', for shoot tips from *in vitro* and *ex vitro* germinated seeds, respectively.

After this first evaluation at 4 wk of culture, shoot tips were subcultured, as medium browned, at 4-wk intervals. Most explants eventually died during subsequent transfers. For 'Irwin', all of them died while after 5 mo of culture, four 'Ataulfo' shoots (out of 67 initial explants) persisted, i.e. around 6% of the explants. Although these shoots showed growth of new leaves, shoot proliferation from the original shoot was never observed (Fig. 1*f*).

**Cotyledonary Node Establishment** Cotyledonary nodes from 'Ataulfo', 'Irwin', 'Gomera-4' and 'Sabre' were placed onto MI2 medium. After 5 wk of culture, no differences in explant survival rate among genotypes were observed, with approximately half of the explants alive for each genotype under the conditions tested (Table 2). At this point, in some of the explants, new shoots appeared from the axils (Fig. 1*g, h*). The number of regenerating explants was significantly affected ($p \leq 0.05$) by the genotype (Table 2). Thus, 'Irwin' showed the lowest regeneration rate (4.0%). The best response was observed for 'Ataulfo' and 'Sabre' with 33.3% and 26.1% of regenerating explants, respectively. 'Gomera-4' displayed an intermediate response with a 13.3% regeneration rate (Table 2). There were no significant differences among genotypes in terms of the number of shoots per regenerating explant, with approximately two shoots per explant (Table 2) and one shoot in each cotyledonary node axil (Fig. 1*h*).

Further experiments were performed to study the possible effect of other plant growth regulators, such as GA or TDZ, on the regeneration from cotyledonary nodes. The effect of GA at the germination step was studied by placing 'Keitt' seeds on GM medium with 0.0, 0.5 or 1.0 mg l$^{-1}$ GA for 3 wk; after that period, cotyledonary node explants were collected and cultured onto MI2 medium. The GA treatment did not affect germination (Table 3), with an average germination rate of about 90%, regardless of the treatment applied. Distinctively from the other genotypes tested, after 3 wk of

**Table 2.** *In vitro* establishment of cotyledonary node explants

| Genotype | Number of explants | Explant survival (% ± S.E.) | Regeneration rate (% ± S.E.) | Number of shoots per regenerating explant (mean ± S.E.) |
|---|---|---|---|---|
| 'Ataulfo' | 21 | 57.1 ± 10.8 a | 33.3 ± 10.3 a | 1.90 ± 0.46 a |
| 'Irwin' | 26 | 69.2 ± 9.0 a | 4.0 ± 3.2 b | 2.07 ± 0.23 a |
| 'Gomera-4' | 15 | 53.3 ± 12.9 a | 13.3 ± 8.8 ab | 1.50 ± 0.50 a |
| 'Sabre' | 23 | 39.1 ± 10.2 a | 26.1 ± 9.2 a | 2.16 ± 0.24 a |

Data was collected 5 wk after the explants were placed on MI2 medium. Different *letters* within the same *column* indicate significant differences ($p \leq 0.05$) among genotypes

**Table 3.** Effect of GA, during seed germination, on the regeneration from mango cv. 'Keitt' cotyledonary nodes

| Treatment | | After 3 wk of culture on GM with the GA treatment | | | | After 5 additional weeks of culture on MI2 (8 wk of culture from the beginning of the experiment) | | |
|---|---|---|---|---|---|---|---|---|
| GA (mg l$^{-1}$) | Number of explants | Germination (% ± S.E) | Regeneration rate (% ± S.E.) | Number of shoots per regenerating explant (mean ± S.E.) | Length of shoots (cm) (mean ± S.E.) | Regeneration rate (% ± S.E.) | Number of shoots per regenerating explant (mean ± S.E.) | Length of shoots (cm) (mean ± S.E.) |
| 0.0 | 20 | 90.0 ± 7.1a | 65.0 ± 10.7a | 1.47 ± 0.15a | 1.40 ± 0.40 a | 70.0 ± 9.7a | 2.00 ± 0.29a | 1.68 ± 0.34a |
| 0.5 | 18 | 80.0 ± 10.3a | 33.0 ± 12.1ab | 1.80 ± 0.20a | 1.60 ± 0.50 a | 40.0 ± 12.6ab | 2.40 ± 0.51a | 1.70 ± 0.48a |
| 1.0 | 20 | 100 ± 0a | 25.0 ± 9.7b | 1.40 ± 0.24a | 1.80 ± 0.60 a | 25.0 ± 9.7b | 1.80 ± 0.24a | 1.48 ± 0.54a |

Different *letters* within a *column* indicate significant differences ($p \leq 0.05$) among treatments

culture on GM, when explants were collected, some of the 'Keitt' cotyledonary nodes already exhibited shoots (Fig. 1*j, k*). Data showed that the number of regenerating explants was reduced as GA concentration increased (Table 3). This decrease was statistically significant ($p \leq 0.05$) when the treatment where GA was not added and the treatment with 1.0 mg l$^{-1}$ GA were compared (Table 3). After 8 wk of culture, 70.0% of the explants in the GA-free treatment showed adventitious shoot regeneration, while in the treatment with 1.0 mg l$^{-1}$ GA, only 25.0% of the explants showed regeneration. The GA treatments tested did not affect the number of shoots per regenerating explant or the length of shoots (Table 3).

The effect of replacing kinetin by TDZ in the MI2 medium was also explored with 'Keitt' cotyledonary nodes. Only the treatment with 3.0 mg l$^{-1}$ slightly improved the average number of shoots per regenerating explant compared with the kinetin treatment (Table 4). Also, the length of the shoots was significantly reduced with this treatment (Table 4). Interestingly, when TDZ was applied (at 2.0 and 3.0 mg l$^{-1}$), explants showing multiple shoot regeneration were observed for the first time (Fig. 1*i*), with 4 up to 9 shoots per regenerating explant (Table 4).

Rooting and acclimatization of rooted shoots was achieved for all three genotypes tested (Fig. 4). The genotype influenced rooting ($p \leq 0.01$) and acclimatization ($p \leq 0.05$) rates. The best rooting results were achieved for 'Ataulfo' and 'Irwin' (78.95% and 60.00%, respectively), and the best acclimatization rates were observed for 'Keitt' and 'Irwin' (83.33% and 78.05%, respectively).

## Discussion

***In Vitro* vs. *Ex Vitro* Germination** Although the same seed disinfection procedure was applied to all genotypes, contamination rates varied among them (Table 1). This result could be explained by the ripening stage of the fruits. Thus, for 'Sabre' where most seeds exhibited contamination (72.0%), fruits were overripe, which could increase the

microbiological load in the fruit pulp and, thus, in the whole fruit (Camataro *et al*. 2018).

For the monoembryonic cultivar 'Irwin', germination rates were significantly higher *in vitro* than *ex vitro* (Table 1). For both polyembryonic genotypes tested ('Ataulfo' and 'Gomera-4'), *in vitro* and *ex vitro* germination rates were similar (Table 1) but the number of germinated embryos per seed was significantly higher when the seeds were germinated *in vitro* (Fig. 2*a*). This is noteworthy since *in vitro* germination of mango mature seeds could improve the efficiency of breeding programs allowing the recovery of more seedlings following directed crosses. In the polyembryonic genotypes, especially those used as rootstocks such as 'Gomera-4', a higher number of germinated embryos could increase the number of available rootstocks for nurseries and growers. SSR analysis of the putative apomictic embryos developed from the maternal tissue showed that all the plants derived from the same seed are genetically identical (Suppl. Figure 1). Some authors have also proposed *ex vitro* shoot tip grafting for rescuing *in vitro* raised hybrids on *in vivo* raised rootstocks (Dinesh *et al*. 2005).

**Shoot Tips** In previous reports (Yang and Lüdders 1993; Thomas and Ravindra 1997; Chandra *et al*. 2004; Samaan *et al*. 2007; Krishna *et al*. 2008; Tetsumura *et al*. 2016), shoot tips and/or stem nodes have been assayed for *in vitro* mango establishment and micropropagation with little success. Major problems are related to latent microbial contamination, excessive polyphenol exudation, plant growth medium browning and explant necrosis. After wounding and/or explant disinfection, secondary metabolism is stimulated, and polyphenols are oxidized. This leads to massive necrosis and explants cannot survive long enough to respond to culture conditions making it difficult to reach any conclusions from those experiments (reviewed by Petri *et al*. 2021).

In order to reduce explant necrosis, we added the antioxidant lipoic acid to MI1 and MI2. Lipoic acid is a sulfur-containing compound involved in several multienzyme

**Table 4.** Effect of TDZ on regeneration of mango cv. 'Keitt' cotyledonary nodes

| Treatment | Explants with shoots (% ± S.E.) | Number of shoots per regenerant explant (mean ± S.E.) | Length of shoots (cm) (mean ± S.E.) | Number of explants with multiple shoot regeneration |
|---|---|---|---|---|
| Kinetin (3.0 mg l$^{-1}$) | 82.35 ± 9.25 a | 2.64 ± 0.34 ab | 2.48 ± 2.07 a | 0 |
| TDZ 1.0 mg l$^{-1}$ | 88.23 ± 7.80 a | 2.13 ± 0.27 ab | 1.01 ± 1.20 ab | 0 |
| TDZ 2.0 mg l$^{-1}$ | 83.33 ± 8.78 a | 1.80 ± 0.31 a | 1.23 ± 1.42 ab | 1 |
| TDZ 3.0 mg l$^{-1}$ | 63.15 ± 11.06 a | 3.75 ± 0.95 b | 0.46 ± 0.68 b | 4 |

Data was collected 5 wk after placing the explants onto MI2 medium. Different *letters* within a *column* indicate significant differences ($p \leq 0.01$) among treatments. A total of 80 cotyledonary nodes, 20 explants per each treatment, were used in this study

**Figure 4.** Rooting and acclimatization of mango shoots excised from cotyledonary nodes. (*a*) Rooting and acclimatization rates (%). Different *letters* within the rooting *columns* indicate significant differences ($p \leq 0.01$). Different *letters* within the acclimatization *columns* indicate significant differences ($p \leq 0.05$). A total of 129 shoots collected from cotyledonary nodes were used in this study. (*b*, *c*) *In vitro* rooted mango shoots. (*d*) Rooted potted plantlets introduced into plastic bags and maintained in a growth chamber during acclimatization. (*e*) Acclimatized plants grown in a greenhouse.
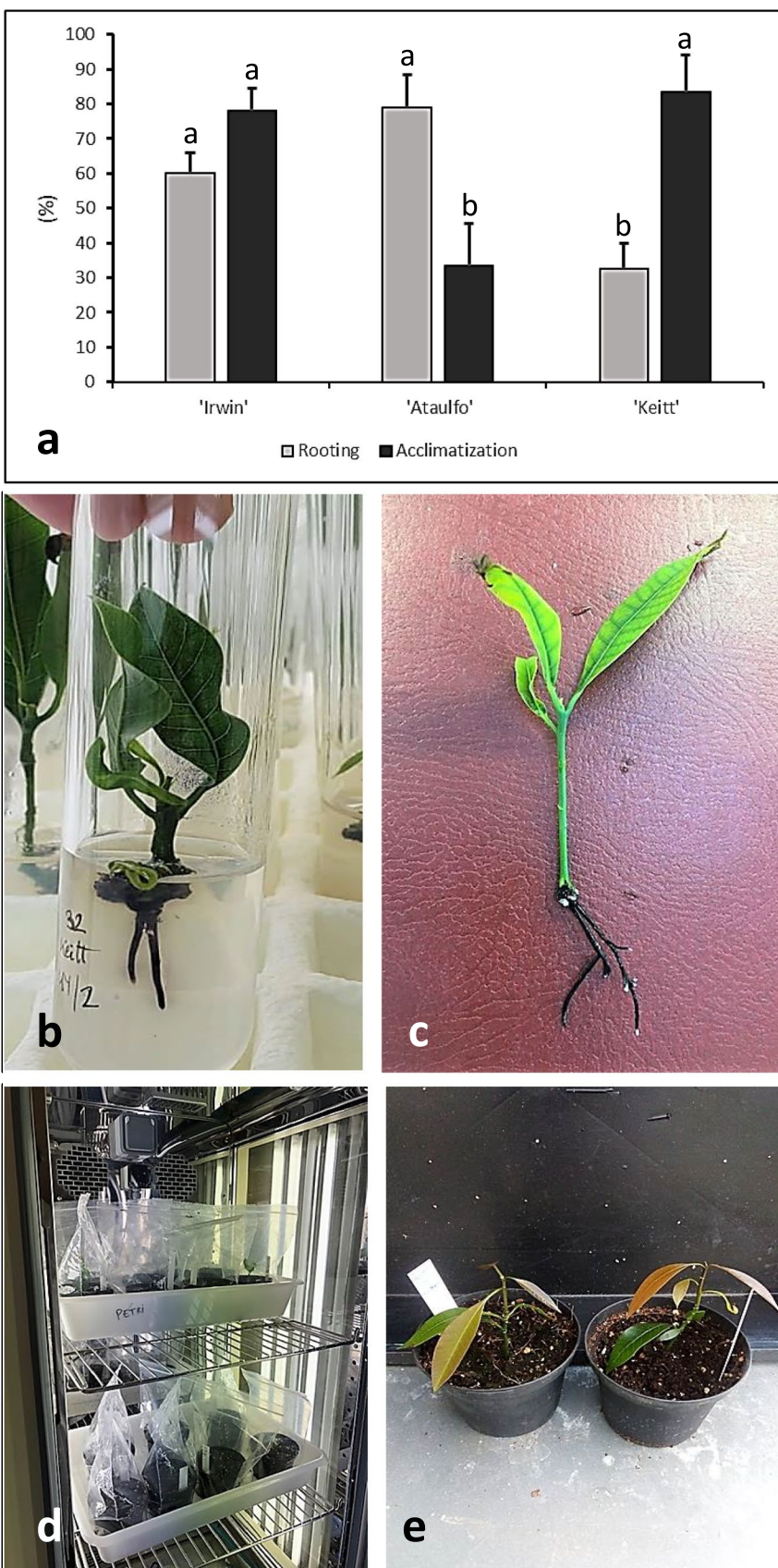
complexes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain keto acid dehydrogenase and glycine decarboxylase (Packer *et al*. 1995). Although its antioxidant effect has been known for several years, lipoic acid is not commonly used in plant tissue culture. However, it has been applied successfully to reduce explant necrosis in tomato, wheat, cotton, soybean and Mexican lime (Dan 2008; Dan *et al*. 2009; Dutt *et al*. 2011). In this work, specific experiments to study the effect of lipoic acid on mango tissues were not performed. Only one concentration (100 mg l$^{-1}$) was assayed since that concentration reduced necrosis in other recalcitrant woody species such as apricot (Isabel M.G. Padilla, personal communication). Future studies should address the effects and optimal concentration of lipoic acid in mango *in vitro* culture.

To the best of our knowledge, there are no reports about mango *in vitro* establishment from shoot tips of *in vitro* germinated mature seeds. Our results demonstrated that the collection of juvenile shoot tips from *in vitro* germinated seeds is preferable than collecting the explants from *ex vitro* germinated seedlings. The contamination rates were significantly reduced, for both genotypes studied, when explants were collected from *in vitro* germinated seeds. Furthermore, explants collected from *in vitro* seedlings showed less necrosis compared with the *ex vitro* collected explants, although the difference was only significant for 'Ataulfo'. This may be explained because *in vitro* materials have a lower microorganism load and lower activity of phenols and oxidative enzymes, which were previously shown to have a high positive correlation with explant necrosis (Krishna *et al*. 2008).

For *in vitro* mango shoot tips or stems establishment, the genotype, the age of the explants, the ontogenic age of donor plants, the period of the year when explants are collected and the disinfection method applied have been described as important factors affecting contamination and necrosis rates. When strong disinfection treatments are applied, contamination is reduced but explant necrosis increases; the use of young shoots reduces contamination but also reduces the explant response and stimulates phenol exudation and necrosis (reviewed by Petri *et al*. 2021). To date, the best results reported on mango *in vitro* shoot establishment involved shoot tips and nodal explants from 2-yr-old mango seedlings ('Terpentine', 'Sabre' and 'Gomera') cultured in a greenhouse (Yang and Lüdders 1993). Explants, collected from May to June, were surface disinfected using procedures similar to those described in this manuscript for the *ex vitro* collected shoot tips, and cultured on a medium consisting of G salts and vitamins (Yang *et al*. 1984) supplemented with 1.0 mg l$^{-1}$ BAP, 1.0 mg l$^{-1}$ zeatin, 2.0 mg l$^{-1}$ N6-(2-isopentenyl) adenine (2iP) and 1.0 mg l$^{-1}$ IAA and 0.5 mg l$^{-1}$ indole butyric acid (IBA). With this procedure, authors described nearly 100% explant survival for both types of explants for all genotypes tested after 4 wk of culture (Yang

and Lüdders 1993). Nevertheless, other studies reported, at best, 50% of explant survival after 4 wk of culture (Thomas and Ravindra 1997; Chandra *et al*. 2004; Samaan *et al*. 2007; Krishna *et al*. 2008). Most frequently, additional information after the first 4 wk of culture was not reported and, when provided, the procedures applied only succeeded to delay microbial contamination and, eventually, high contamination levels caused massive death (Chandra *et al*. 2004; Samaan *et al*. 2007). These results on explant survival at 4 wk of culture are in line with our findings for 'Ataulfo' (approx. 50% of explant survival). However, for 'Irwin', we observed higher levels of necrosis (81%), probably due to the effect of the genotype.

Regarding *in vitro* shoot multiplication, very little information is available. Similar to our results, other authors have described shoot growth until 3–4 leaf primordial stage after approx. 16 wk of culture in 6% of the explants, without any proliferation of axillary shoots (Chandra *et al*. 2004; Tetsumura *et al*. 2016). To our knowledge, the production of new lateral shoots has only been reported in two publications with 3.7 and 1.8 lateral shoots per initial shoot for 'Hindy Sinnara' (Samaan *et al*. 2007) and 'Irwin' (Tetsumura *et al*. 2016), respectively. However, further reports replicating these results have not been published so far.

***Cotyledonary Nodes*** Similarly to mango, cashew (*Anacardium occidentale* L.), another species in the Anacardiaceae, is recalcitrant to micropropagation. Since it is difficult to obtain surviving explants from mature cashew plants grown in the field, explants from seedlings obtained by *in vitro* germination are the most suitable for cashew micropropagation (Nanti *et al*. 2020). The use of cotyledonary nodes has been shown appropriate for cashew *in vitro* multiplication, with up to 78% regeneration rate and a yield of 9 shoots per regenerating explant (Nanti *et al*. 2020). Therefore, in this study, we explored the use of this explant source in mango. In our case, regeneration was achieved for all the genotypes tested, although regeneration rates were highly genotype-dependent. The application of the synthetic cytokinin TDZ induced a high-frequency shoot regeneration from the axils of cotyledonary nodes in other woody plants, such as *Anacardium occidentale*, *Cassia sophera* and *Sterculia urens* (Hussain *et al*. 2007; Parveen and Shahzad 2010; Nanti *et al*. 2020). In our case, the TDZ treatments did not appear to improve the regeneration percentage from cv. 'Keitt' mango cotyledonary nodes (Table 4). However, for the first time, we observed a few explants with multiple shoot regeneration (Fig. 1*i*), displaying a high-frequency regeneration pattern similar to that reported in other species when TDZ was applied (Hussain *et al*. 2007; Parveen and Shahzad 2010; Nanti *et al*. 2020). In our study, data analyses did not reveal statistically significant differences in the frequency of explants with multiple shoot regeneration among treatments, probably due to the small sample size. When this study was performed, there were no more mature seeds of 'Keitt'

🖄 Springer

available and, hence, the number of explants per treatment could not be increased. Further experiments with more explants and additional TDZ treatments should be tested in future studies to validate the hypothesis of TDZ as a high-frequency shoot regeneration inducer in mango *via* cotyledonary node explants.

GA is an important endogenous growth regulator that has profound and diverse effects on plant growth and development. Specific roles of GA include the induction of seed germination and the promotion of hypocotyl and stem elongation (Peng and Harberd 2002). Several studies have reported that the exogenous addition of GA in plant tissue culture stimulated bud organogenesis or shoot elongation in different plant species (Pérez-Tornero *et al*. 1999; Shen *et al*. 2013; Navarro-García *et al*. 2016; Huh *et al*. 2017; Lee and Pijut 2017). However, in some cases, GA reduced or prevented the formation of roots, shoots or somatic embryos (Srejovic and Neskovic 1985; Peng and Harberd 2002). In our study, GA, applied during *in vitro* mango seed germination, did not affect the germination rate and reduced the consequent regeneration from cotyledonary nodes. Srejovic and Neskovic (1985) have reported that GA, in combination with BA, inhibited bud induction from buckwheat cotyledons and the initial GA treatment had a long-term inhibition effect from which the tissue recovered only in the fifth subculture. Some differences in the inhibition or promotion of adventitious root and shoot formation by GAs might be due to the fact that GAs inhibit meristemoid initiation (Peng and Harberd 2002).

The two-step mango rooting procedure described by Ara *et al*. (1998) was suitable for shoots of all the three genotypes tested in our study. Authors suggested that shoots acquire competence with auxins (5.0 mg l$^{-1}$ IBA) during the first 24 h step and induce rooting on the auxin-free rooting medium during the second step, whereas the continuous presence of auxin suppresses rooting. They described rates of around 90% for *in vitro* rooting of microshoots excised from plantlets obtained from nucellar somatic embryos of 'Amrapali'. Our results confirmed the validity of their method and pointed out the genotype dependence of the process. Similarly, our results showed the acclimatization step to be highly influenced by the genotype. To increase rooting and acclimatization rates, the application of biostimulants should be studied in future experiments. In peach (*Prunus persica*), another recalcitrant fruit tree species, the use of volatile compounds of the fungus *Cladosporium sphaerospermum* enhanced *in vitro* root growth and subsequent plantlet acclimatization (Ricci *et al*. 2020).

# Conclusions

In this work, a procedure for *in vitro* establishment and micropropagation of mango juvenile material has been achieved. Although shoot tips from seedlings failed as the source of explants, *in vitro* establishment of cotyledonary nodes and micropropagation of shoots excised from the cotyledonary nodes were accomplished. Our results show the cotyledonary node as a feasible explant for an efficient regeneration system for mango mass propagation. Further research will be focused on factors affecting the regeneration from mango cotyledonary nodes and on the improvement of microshoot rooting and acclimatization efficiency. In this respect, the application of different combinations of plant growth regulators, the addition of nanoparticles (Kim *et al*. 2017) and/or polyphenol oxidases inhibitors (Chimvaree *et al*. 2020; Moon *et al*. 2020) and the use of biostimulants (Soumare *et al*. 2021) should be considered in future experiments.

The ability to consistently regenerate plants from mango cotyledonary nodes has a number of practical applications. The method allows rapid shoot multiplication as numerous shoots are produced from each seed. This is especially useful in the case of polyembryonic mangoes, where most regenerated shoots derive from true-to-type apomictic embryos. Shoots regenerated from the zygotic explant can be easily identified with molecular techniques such as SSR analyses. Other potential application includes the genetic improvement of mango through the introduction of engineered genes, coupling this regeneration system with genetic transformation techniques.

We also found that *in vitro* seed germination in mango increases the germination rate and allows the recovery of more seedlings compared with *ex vitro* conditions. These two features could improve the efficiency of mango breeding programs and, in the case of polyembryonic genotypes, increase the number of clonal progenies. Overall, our results represent a qualitative advancement for *in vitro* propagation of mango, a recalcitrant species in which advances so far have been scarce compared to other fruit crops.

## Declarations

**Competing Interests** The authors declare no competing interests.

# References

Ara H, Jaiswal U, Jaiswal VS (1998) Rooting of microshoots of *Mangifera indica* L. cv Amrapali. Curr Sci 74:240–242

Chandra R, Padaria JC, Shubhra S (2004) Factors influencing *in vitro* establishment of mango shoot buds. Indian J Plant Physiol 9:136–144

Chimvaree C, Cumsingnok T, Wongs-Aree C *et al* (2020) Substrate reactivity of polyphenol oxidase and browning inhibition of fresh-cut 'nam dok mai si-thong' mangoes by protein-based sericin coating. Hortic J 89:537–544. https://doi.org/10.2503/hortj.UTD-154

Dan Y (2008) Biological functions of antioxidants in plant transformation. Vitr Cell Dev Biol - Plant 44:149–161. https://doi.org/10.1007/s11627-008-9110-9

Dan Y, Armstrong CL, Dong J *et al* (2009) Lipoic acid-an unique plant transformation enhancer. Vitr Cell Dev Biol - Plant 45:630–638. https://doi.org/10.1007/s11627-009-9227-5

Dinesh M, Halesh G, Sahijram L *et al* (2005) *In vitro* hybrid embryo rescue in mango (*Mangifera indica* L.) breeding. Indian J Hortic 62:235–237

dos Santos Camataro FO, de Aquino Santana LCL, Carnelossi MAG *et al* (2018) Impact of edible coatings based on cassava starch and chitosan on the post-harvest shelf life of mango (*Mangifera indica*) 'tommy atkins' fruits. Food Sci Technol 38:86–95. https://doi.org/10.1590/1678-457x.16417

Dutt M, Vasconcellos M, Grosser JW (2011) Effects of antioxidants on *Agrobacterium*-mediated transformation and accelerated production of transgenic plants of Mexican lime (*Citrus aurantifolia* Swingle). Plant Cell Tissue Organ Cult 107:79–89. https://doi.org/10.1007/s11240-011-9959-x

FAOSTAT (2021) FAOSTAT database. http://www.fao.org/faostat/en/. Accessed 11 Jan 2021

Huh YS, Lee JK, Nam SY (2017) Effect of plant growth regulators and antioxidants on *in vitro* plant regeneration and callus induction from leaf explants of purple passion fruit (*Passiflora edulis* Sims). J Plant Biotechnol 44:335–342. https://doi.org/10.5010/JPB.2017.44.3.335

Hussain TM, Chandrasekhar T, Gopal GR (2007) High frequency shoot regeneration of *Sterculia urens* Roxb. an endangered tree species through cotyledonary node cultures. African J Biotechnol 6:1643–1649

Kim DH, Gopal J, Sivanesan I (2017) Nanomaterials in plant tissue culture: the disclosed and undisclosed. RSC Adv 7:36492–36505. https://doi.org/10.1039/c7ra07025j

Krishna H, Sairam RK, Singh SK *et al* (2008) Mango explant browning: effect of ontogenic age, mycorrhization and pre-treatments. Sci Hortic (amsterdam) 118:132–138. https://doi.org/10.1016/j.scienta.2008.05.040

Lee JH, Pijut PM (2017) Adventitious shoot regeneration from *in vitro* leaf explants of *Fraxinus nigra*. Plant Cell Tissue Organ Cult 130:335–343. https://doi.org/10.1007/s11240-017-1228-1

Moon KM, Kwon E Bin, Lee B, Kim CY (2020) Recent trends in controlling the enzymatic browning of fruit and vegetable products. Molecules 25:2754. https://doi.org/10.3390/molecules25122754

Mukherjee SK, Litz RE (2009) Introduction: botany and importance. In: Litz RE (ed) The mango: botany, production and uses, 2nd Editio. CAB International, pp 1–18

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nanti BTJ-I, Gnamien YG, Kone T *et al* (2020) *In vitro* regeneration of Anacardium occidentale from shoot tip and basal part. Int J Environ Agric Biotechnol 5:621–630. https://doi.org/10.22161/ijeab.53.14

Navarro-García N, Morte A, Pérez-Tornero O (2016) *In vitro* adventitious organogenesis and histological characterization from mature nodal explants of *Citrus limon*. Vitr Cell Dev Biol - Plant 52:161–173. https://doi.org/10.1007/s11627-015-9743-4

Packer L, Witt EH, Tritschler HJ (1995) Alpha-lipoic acid as a biological antioxidant. Free Radic Biol Med 19:227–250. https://doi.org/10.1016/0891-5849(95)00017-R

Parveen S, Shahzad A (2010) TDZ-induced high frequency shoot regeneration in *Cassia sophera* Linn. via cotyledonary node explants. Physiol Mol Biol Plants 16:201–206. https://doi.org/10.1007/s12298-010-0022-x

Peng J, Harberd NP (2002) The role of GA-mediated signalling in the control of seed germination. Curr Opin Plant Biol 5:376–381. https://doi.org/10.1016/S1369-5266(02)00279-0

Pérez-Hernández JB, Grajal-Martín MJ (2011) *In vitro* culture of immature zygotic mango embryos and plantlet development. HortScience 46:1528–1532. https://doi.org/10.21273/hortsci.46.11.1528

Pérez-Tornero O, Burgos L, Egea J (1999) Introduction and establishment of apricot in vitro through regeneration of shoots from meristem tips. Vitr Cell Dev Biol - Plant 35:249–253

Petri C, Litz RE, Singh SK, Hormaza JI (2021) *In vitro* culture and genetic transformation in mango. In: Kole C (ed) The mango genome. Springer Nature Switzerland AG, Cham, pp 131–153. https://doi.org/10.1007/978-3-030-47829-2_8

Ricci A, Sabbadini S, Prieto H *et al* (2020) Genetic transformation in peach (*Prunus persica* l.): challenges and ways forward. Plants 9:1–31. https://doi.org/10.3390/plants9080971

Samaan M, Wafaa H, Rawash M (2007) Factors affecting in vitro establishment and multiplication of some mango (Mangifera indica L.) rootsotcks. J Biol Chem Environ Sci 2:79–93. https://doi.org/10.13140/RG.2.2.27066.70080

Sharma KK, Lavanya M, Anjaiah V (2006) *Agrobacterium*-mediated production of transgenic pigeonpea (*Cajanus cajan* L. Millsp.) expressing the synthetic Bt *cry*1Ab gene. Vitr Cell Dev Biol - Plant 42:165–173. https://doi.org/10.1079/IVP2005730

Shen X, Orbović V, Dutt M *et al* (2013) Direct shoot organogenesis in Murraya paniculata (L.) Jack: a prerequisite for genetic transformation. HortScience 48:938–941. https://doi.org/10.21273/hortsci.48.7.938

Soumare A, Diédhiou AG, Arora NK *et al* (2021) Potential role and utilization of plant growth promoting microbes in plant tissue culture. Front Microbiol 12:649878. https://doi.org/10.3389/fmicb.2021.649878

Souza FVD, Rebouças CC, Souza EH, Peixoto CP (2020) *In vitro* conservation of mango (*Mangifera indica* L.) ubá and carlota cvs. through culturing immature embryos. An Acad Bras Cienc 92:1–11. https://doi.org/10.1590/0001-3765202020190400

Srejovic V, Neskovic M (1985) Effect of gibberellic acid on organogenesis in buckwheat tissue culture. Biol Plant 27:432–437

Tetsumura T, Sakota T, Nagano H *et al* (2016) Plant regeneration from nodal explants of "Irwin" mango seedlings. Acta Hortic 1113:127–134. https://doi.org/10.17660/ActaHortic.2016.1113.18

Thomas P, Ravindra MB (1997) Shoot tip culture in mango: influence of medium, genotype, explant factors, season and decontamination treatments on phenolic exudation, explant survival and axenic culture establishment. J Hortic Sci 72:713–722. https://doi.org/10.1080/14620316.1997.11515563

Viruel MA, Escribano P, Barbieri M *et al* (2005) Fingerprinting, embryo type and geographic differentiation in mango (*Mangifera indica* L., Anacardiaceae) with microsatellites. Mol Breed 15:383–393. https://doi.org/10.1007/s11032-004-7982-x

Viruel MA, Hormaza JI (2004) Development, characterization and variability analysis of microsatellites in lychee (*Litchi chinensis* Sonn., Sapindaceae). Theor Appl Genet 108:896–902. https://doi.org/10.1007/s00122-003-1497-4

Yang Z, Lüdders P (1993) Effect of growth regulators and media on *in vitro* shoot tipi culture of different cultivars of mango (*Mangifera indica* L.). Acta Hortic 341:240–247. https://doi.org/10.5829/idosi.wasj.2014.31.05.14332

Yang ZH, Hu NY, Lu GM (1984) *In vitro* shoot tips culture of peach. Acta Univ Sept Occ Agri 1:13–19

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.