
# Cryopreservation of vanilla (*Vanilla planifolia*) root-tips: A new alternative for *in vitro* long-term storage of its germplasm

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***Abstract***

*Vanilla planifolia* (Orchidaceae) is the natural source of vanillin, which is the most widely appreciated flavor compound in the world. Although vanilla is cultivated throughout the tropics, their natural distribution areas are being severely reduced due to the anthropogenic impact. As a result, *ex situ* preservation actions are necessary to safeguard the threatened diversity of this species.

Cryopreservation of vanilla shoot-tips has been previously reported using the droplet-vitrification technique. However, survival and plant regeneration following this protocol were low and little reproducible.

In this study, we evaluated a new alternative for cryopreservation of vanilla germplasm by using root-tips as explants and following the droplet-vitrification protocol. Maximum survival (~60%) and further regeneration (~40%) were obtained by preconditioning root-tips isolated from *in vitro* propagated plants on semisolid MS with 0.3 M sucrose (1 day), exposing to loading solution consisting of 0.4 M sucrose + 2 M glycerol (30 min) followed by glycerol-sucrose plant vitrification solution PVS3 (60 min in ice), and direct plunging into liquid nitrogen in droplets of PVS3 placed on aluminum foils strips. Tissues were rewarmed by plunging the aluminum foils directly in liquid MS enriched with 1.2 M sucrose (15 min) at room temperature. rowth recovery and induction of buds were efficiently achieved by culturing cryostored root-tips on MS added with 1 mg $L^{-1}$ KIN or BAP. Plant regeneration was achieved by transferring the induced buds to MS media.

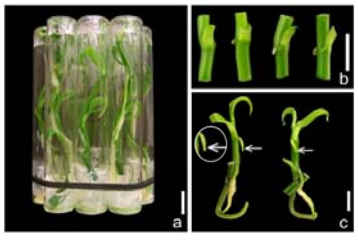
This protocol has great potential for long-term conservation of *V. planifolia* germplasm and of other vanilla relatives.

## *In vitro culture: Adventitious shoot regeneration from root-tips*

Table 1. Effect of type, concentration and combination of cytokinins and auxins added to MS medium on adventitious shoot regeneration from *Vanilla planifolia* root-tips.

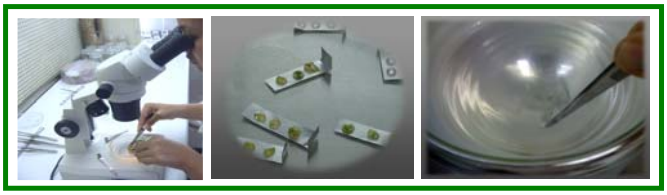
| KIN [mg $L^{-1}$] | IBA [mg $L^{-1}$] | NAA [mg $L^{-1}$] | Regeneration (%)[1] | BAP [mg $L^{-1}$] | IBA [mg $L^{-1}$] | NAA [mg $L^{-1}$] | Regeneration (%)[1] |
|---|---|---|---|---|---|---|---|
| - | - | - | 0.0±0.0d | - | - | - | 0.0±0.0d |
| 0.5 | - | - | 73.3±6.7a | 0.5 | - | - | 66.7±8.8a |
| 1.0 | - | - | 80.0±5.8a | 1.0 | - | - | 83.3±6.7a |
| 3.0 | - | - | 63.3±8.8a | 3.0 | - | - | 63.3±8.8a |
| 0.5 | 0.01 | - | 60.0±11.6a | 0.5 | 0.01 | - | 63.3±8.8a |
| 1.0 | 0.01 | - | 66.7±8.8a | 1.0 | 0.01 | - | 73.3±6.7a |
| 3.0 | 0.01 | - | 40.0±10.0ab | 3.0 | 0.01 | - | 43.3±8.8ab |
| 0.5 | 0.10 | - | 10.0±5.8c | 0.5 | 0.10 | - | 23.3±8.8b |
| 1.0 | 0.10 | - | 13.3±6.7c | 1.0 | 0.10 | - | 40.0±10.0ab |
| 3.0 | 0.10 | - | 16.7±8.8bc | 3.0 | 0.10 | - | 6.7±3.3c |
| 0.5 | - | 0.01 | 60.0±10.0a | 0.5 | - | 0.01 | 56.7±6.7a |
| 1.0 | - | 0.01 | 70.0±10.0a | 1.0 | - | 0.01 | 60.0±5.8a |
| 3.0 | - | 0.01 | 43.3±8.8a | 3.0 | - | 0.01 | 40.0±5.8ab |
| 0.5 | - | 0.10 | 6.7±3.3c | 0.5 | - | 0.10 | 0.0±0.0d |
| 1.0 | - | 0.10 | 23.3±8.8abc | 1.0 | - | 0.10 | 6.7±3.3c |
| 3.0 | - | 0.10 | 20.0±5.8abc | 3.0 | - | 0.10 | 0.0±0.0d |

[1]Measured as the percentage of root-tips that displayed shoot organogenesis after 120 days of culture initiation. Values represent mean ± SE. Different letters within a column denote significant differences according to Duncan´s multiple comparison test ($P$<0.05).




(a) *In vitro* mother-plants; (b) Plantlets obtained after 60 days of microcutting culture on MS free of plant growth regulators; (c) Plantlets regenerated 30 days after microcutting subculture, with young roots (indicated with arrows) used as source of explants for cryopreservation experiments. Bars represent 1 cm.

## *Cryopreservation of vanilla root-tips by Droplet-Vitrification*

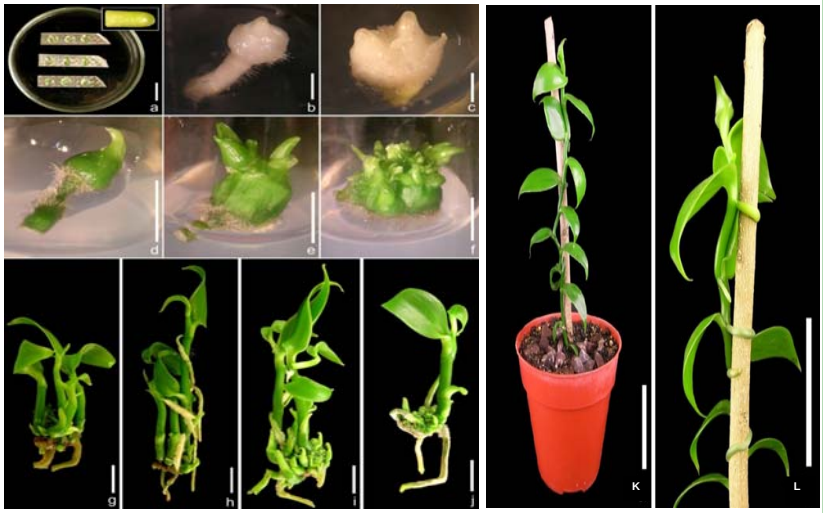


## *Post-cryopreservation recovery and plant regeneration of vanilla root-tips*

Table 2. Effect of preconditioning, loading treatment and different exposure duration to vitrification solution (PVS3) on survival (Surv.) and regrowth (Reg.) of non-cryostored (-LN) and cryostored (+LN) *V. planifolia* root-tips, using droplet vitirfication approach and recovery culture on media with KIN or BAP (1 mg $L^{-1}$).

| Treatments | MS + KIN 1 mg $L^{-1}$ | | | | MS + BAP 1 mg $L^{-1}$ | | | |
|---|---|---|---|---|---|---|---|---|
| | -LN | | +LN | | -LN | | +LN | |
| | Surv. | Reg. | Surv. | Reg. | Surv. | Reg. | Surv. | Reg. |
| Control | 97 a | 80 a | 0 g | 0 d | 93 a | 83 a | 0 g | 0 d |
| Preconditioning | 93 a | 83 a | 0 g | 0 d | 97 a | 77 a | 0 g | 0 d |
| Loading | 93 a | 77 a | 0 g | 0 d | 90 ab | 80 a | 0 g | 0 d |
| PVS3 30 min | 83 abc | 67 a | 37 e | 23 b | 90 ab | 70 a | 17 f | 10 c |
| PVS3 60 min | 87 abc | 70 a | 60 cd | 43 ab | 83 abc | 73 a | 57 d | 37 ab |
| PVS3 90 min | 63 bcd | 43 ab | 23 e | 10 c | 60 cd | 50 ab | 30 e | 7 cd |

Values represent mean of three replicates. Means with the same letter within "survival" and "regrowth" columns are not significantly different according to Duncan´s multiple comparison test ($P$<0.05).




Regeneration from cryostored *V. planifolia* root-tips following droplet-vitrification technique. Shoot bud formation and plant regeneration from root-tips cultured on MS + KIN 1 mg $L^{-1}$, after 45, 60, and 120 days, respectively (d, e, f). Shoot bud formation and plant regeneration from root-tips cultured on MS + BAP 1 mg $L^{-1}$, after 45, 60, 90, and 120 days, respectively (g, h, i, j). *In vitro* regenerated plant transplanted to pot and hardened in the greenhouse conditions after 5 months of *ex vitro* transference. Bars represent 1 cm in a, g-j; 2 mm in b, c; 5 mm in d-f; 5 cm in k, l.