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# SOMATIC EMBRYOGENESIS IN GRAPE (*VITIS VINIFERA* L.) CV. TAS-A-GANESH FROM LEAF EXPLANTS

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## SUMMARY

**Embryogenic calli developed in the leaf discs of field grown grapevine (*Vitis vinifera* L.) cv. Tas-A-Ganesh, cultured on MS medium supplemented with various combinations of BAP (0.0-17.6 µM), TDZ (2.25-4.5 µM) and NOA (5-25.00 µM). Among these combinations, 17.6 µM BAP+4.5 µM TDZ+20 µM NOA combination was the best for embryo production when transferred to MS medium supplemented with 2.2 µM BAP +1 µM IAA (where 26% of calli showed embryogenesis). Somatic embryos were photographed and were confirmed by microtomy. These embryos germinated on hormone-free MS medium. Percentage of germination was however, very poor (9.0%).**

***Key words*: Callus, somatic embryos, *Vitis vinifera* L.**

## INTRODUCTION

Somatic embryogenesis is one of the requisites for successful genetic manipulation of grape by current engineering strategies or the selection of stress tolerant clones from large cell populations. Development of this technology might also be used for the mass propagation of new plant introductions. Mullins and Srinivasan (1976) first reported somatic embryogenesis in *V. vinifera*. It was achieved from callus derived from several tissues, such as anther, unfertilized ovule, ovaries, stem and leaf. The possibility of inducing embryogenic cell masses was suggested in anther (Hirabayashi and Akihama 1982) and leaf (Hirabayashi 1985) callus cultures. When leaf tissue is used, the general mode of regeneration has been via organogenesis (Hirabayashi 1985, Stamp *et al.* 1990). Using leaf discs (Matsuta 1992) and tendrils (Salunkhe *et al.* 1997) of grape, somatic embryogenesis at low frequency has been reported.

The technique of somatic embryogenesis has been improved in several genotypes and among various types of explants (Gray 1995, Perl and Eshdat 1998, Martinelli and Gribaudo 2001). Efficiencies in most agronomically important genotypes need to be improved. Response among cultivated genotypes varies greatly. Investigations on somatic embryogenesis in grape should be continued to achieve breakthrough in propagation. Grape (*V. vinifera* L.) cv. Tas-A-Ganesh is a recent promising cultivar widely grown in Maharashtra, India. Its fruits are being exported to Europe and Arabian countries. No protocol is available for somatic embryogenesis and emblings production from leaf discs of *Vitis vinifera* L. cv. Tas-A-Ganesh. A protocol for the induction of somatic embryogenesis and ambling production from the leaf derived callus of *Vitis vinifera* L. cv. Tas-A-Ganesh has been developed in the present investigation.

## MATERIALS AND METHODS

Twigs of field-grown grapevines (*V. vinifera* L.) cv. Tas-A-Ganesh were obtained from the orchard of National

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Research Center for grapes, Manjari farm, Pune, Maharashtra, India. Third to sixth leaves from the shoot tip were selected for investigations. Leaves were kept in running water for 30 min, treated with 0.5% Teepol and then washed with tap water. Explants were treated with 0.1% Bavistin for 15 min., rinsed with sterile distilled water twice and then treated with 0.1% $HgCl_2$ (w/v) for 15 min. The treated explants were rinsed with sterile distilled water four times and treated with 0.8% sodium hypochlorite. These were again washed with sterile distilled water four times and leaf discs with main veins were excised with cork borer (10 mm diameter).

Callus induction was carried out on MS basal medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% Difco Bacto Agar. Plant growth regulators (PGRs) were added and pH was adjusted to 5.8 before autoclaving. Culture tubes containing medium were autoclaved at 120 $^0$C and 15 lbs pressure for 20 minutes. The lower surface of the leaf discs was in contact with the culture medium. Inoculated tubes were incubated at 25+2 $^0$C, light intensity of 50 µE $m^{-2}s^{-1}$ and a 16/8-h (light/dark) photoperiod for 30 days.

Calli with embryogenic potential were induced on media supplemented with different combinations of BAP (0.0-17.6 µM), TDZ (2.25-4.5 µM) and NOA (5.0-25.0 µM). Calli were subcultured on the medium supplemented with 2.2 µM BAP and 1 µM IAA and incubated for 30 days at the same temperature, light intensity and photoperiod. Somatic embryos were observed and counted under the microscope. These were confirmed through microscopic evaluation of sections obtained from fixed and embedded materials stained with 1.0% safranin, 1.0% fast green and 1.0% haematoxylin. Stained preparations were observed and photographed under the Olympus BX40 phase contrast microscope. Somatic embryos were transferred on the hormone-free MS medium for germination. Emblings were transferred to small pots containing soilrite and sand in the proportion of 1:1 and hardened at 80-85% humidity in a humidity chamber and then in the green house. Finally, plantlets were transferred to field conditions.

## RESULTS AND DISCUSSION

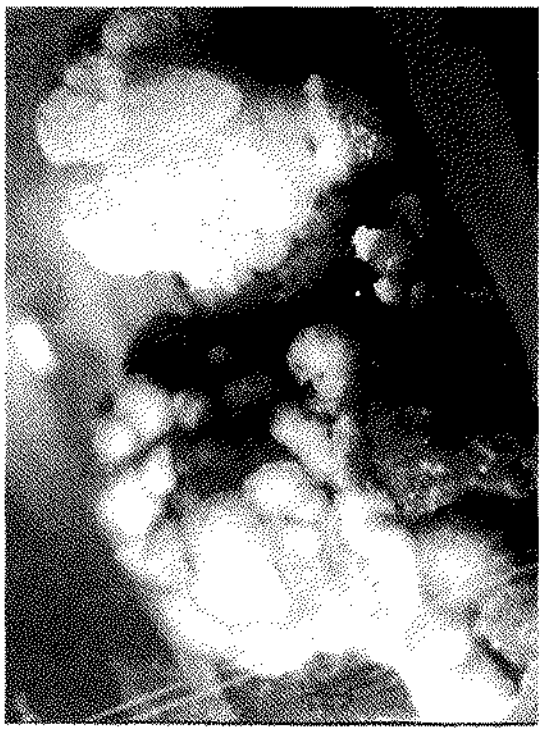
Matsuta (1989) reported that leaves of grape treated with 1.0 µM 2,4-D led to the production of numerous embryoids. Content of BA in the medium is conducive for formation of embryonic callus. NAA in media is important for embryoid production in some genotypes of *Vitis* and 1 mg $l^{-1}$ 2,4-D with only 1 mg $l^{-1}$ BA were effective for embryonic callus production (Marchenko 1991). Somatic embryos were produced on medium containing 10.7 µM NAA and 0.9 µM BA, on callus derived from immature leaf laminae and petioles of *V. rotundifolia* cv. Regale and Fry (Robacker 1993). Torregrosa *et al.* (1995) reported that leaf explants of 3 hybrids cultured on MS medium supplemented with 5 µM 2,4-D, showed strong growth and callogenesis. When this actively proliferating entire embryonic callus was transferred to media supplemented with 5 µM IAA and 1.1 µM BA, it lead to formation of embryos via callus. High frequency somatic embryo induction was obtained from leaf explants on a modified NN 69 medium supplemented with 20 µM NOA and 4 µM TDZ in grapevine cv. Seyval Blanc (Harst 1995). Miaja *et al.* (1997) cultured leaves and petiole explants of grape cv. Barbera, Nebbiolo and Appricot on NN medium supplemented with 1.1 mg $l^{-1}$ 2,4-D + 1.2 mg $l^{-1}$ 4-C PPU (Forchlorfenuron) in the dark at 24 $^0$C. After 40 days, explants were transferred to NN medium supplemented with 0.2 mg $l^{-1}$ 2,4-D. This lead to embryonic calli production. When these were transferred to NN medium without growth regulators, isolated embryos developed.

Our results suggest that embryogenic potential of calli was initiated after 15 days of inoculation and considerable amounts of calli developed at different combinations of PGRs. On MS medium supplemented with 2.2 µM BAP and 1.0 µM IAA, these led to differentiation of somatic embryos after 30 days of subculture (Table 1, Fig. 1a, b, c, d). Different stages of somatic embryos viz. globular, heart-shaped and cotyledonary, were observed (Fig.1b, c, d). These embryos were fixed, dehydrated, embedded in and sectioned using microtome. These were stained and confirmed (Fig. 2a, b, c, d). Embryoids germinated when transferred to hormone-free MS medium. These results (Table 1, Fig. 1) showed that calli produced with lower concentrations (2.25 µM) of TDZ and (5.0 µM) NOA in combination with 4.4, 8.8, 17.6 µM BAP, were unable to induce somatic embryogenesis in grape. However, calli produced with 4.5 µM TDZ in combination with 10, 20 and 25 µM NOA could induce somatic embryogenesis. Thus, somatic

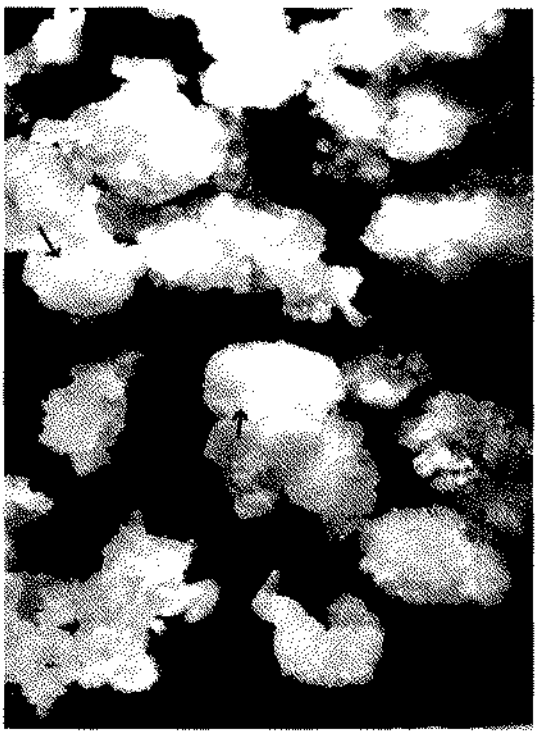
**Table 1.** Effect of BAP, TDZ and NOA on the Somatic Embryogenesis in grape (*V. Vinifera* L.) cv. Tas-A-Ganesh

| BAP μM | TDZ μM | NOA μM | % of leaf discs producing calli | % of calli inducing somatic embryos when transferred to 2.2 μM BAP+1.0 μM IAA |
|---|---|---|---|---|
| 0.0 | 2.25 | 5.0 | 10.0 | 0.0 |
| | | 10.0 | 14.0 | 0.0 |
| | | 20.0 | 18.0 | 0.0 |
| | | 25.0 | 22.0 | 0.0 |
| | 4.5 | 5.0 | 32.0 | 0.0 |
| | | 10.0 | 38.0 | 2.0 |
| | | 20.0 | 40.0 | 12.0 |
| | | 25.0 | 52.0 | 6.0 |
| 4.4 | 2.25 | 5.0 | 22.0 | 0.0 |
| | | 10.0 | 62.0 | 6.0 |
| | | 20.0 | 66.0 | 18.0 |
| | | 25.0 | 70.0 | 8.0 |
| | 4.5 | 5.0 | 44.0 | 0.0 |
| | | 10.0 | 78.0 | 6.0 |
| | | 20.0 | 84.0 | 18.0 |
| | | 25.0 | 86.0 | 14.0 |
| 8.8 | 2.25 | 5.0 | 36.0 | 0.0 |
| | | 10.0 | 80.0 | 12.0 |
| | | 20.0 | 88.0 | 20.0 |
| | | 25.0 | 90.0 | 16.0 |
| | 4.5 | 5.0 | 56.0 | 0.0 |
| | | 10.0 | 90.0 | 16.0 |
| | | 20.0 | 92.0 | 24.0 |
| | | 25.0 | 92.0 | 18.0 |
| 17.6 | 2.25 | 5.0 | 46.0 | 0.0 |
| | | 10.0 | 92.0 | 18.0 |
| | | 20.0 | 94.0 | 24.0 |
| | | 25.0 | 94.0 | 20.0 |
| | 4.5 | 5.0 | 62.0 | 0.0 |
| | | 10.0 | 94.0 | 20.0 |
| | | 20.0 | 96.0 | 26.0 |
| | | 25.0 | 86.0 | 16.0 |

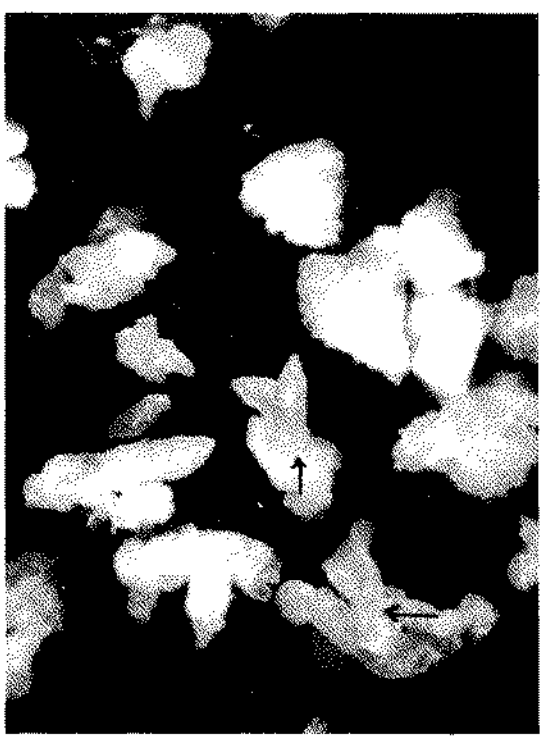
Values are expressed as percentage of cultures of 50 cultures



1a



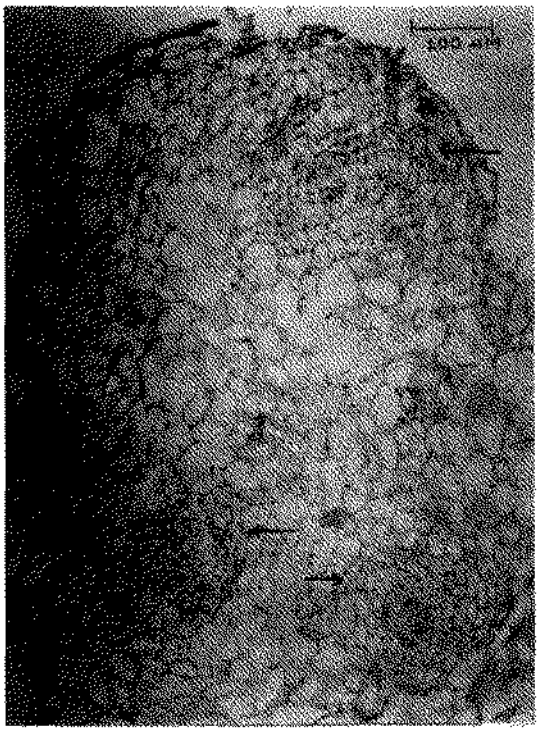
1b



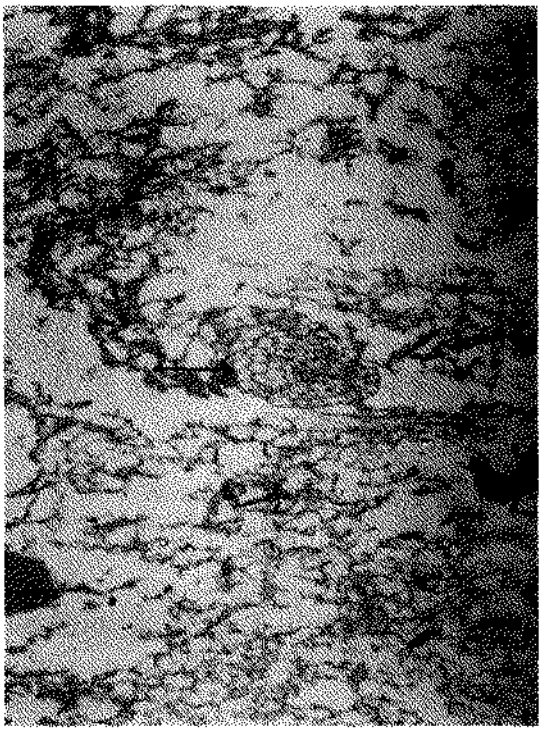
1c

1d

**Fig.1-a. Globular stage of somatic embryos (Scale bar -1mm) or (X 9.5); Fig.1-b. Separated globular embryo (Scale bar -1mm) or (X 9.5); Fig.1-c. Heart shaped stage (Scale bar -1mm) or (X 9.5); Fig.1-d. Cotyledonary stage (Scale bar -1mm) or (X 9.5).**



2a



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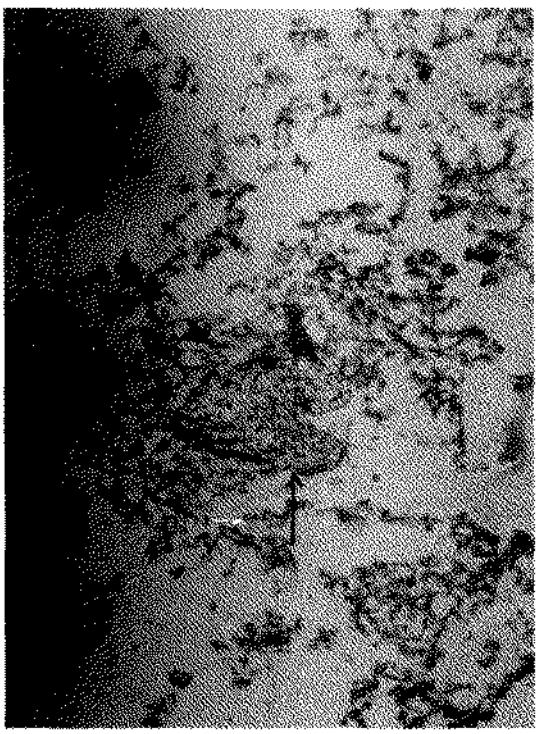

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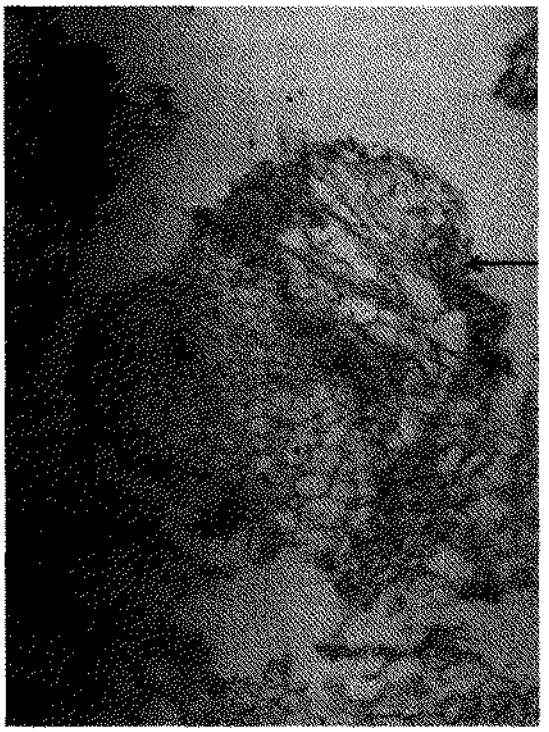


2d

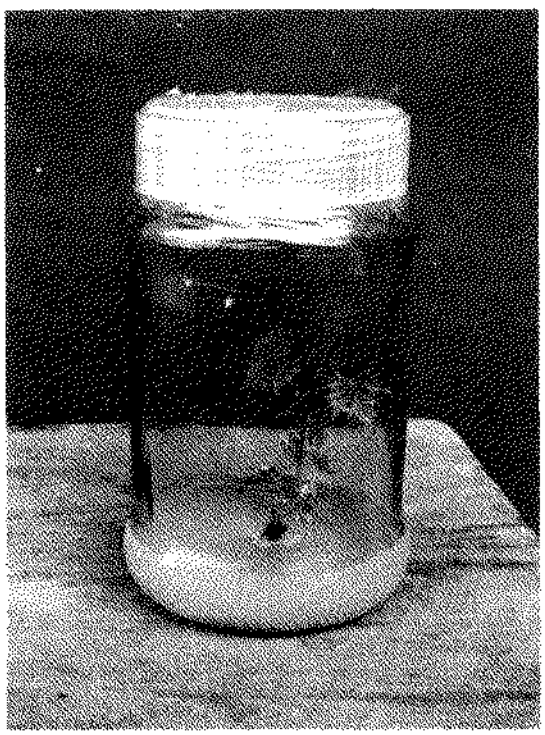
**Fig.2-a. Initiation of somatic embryos under phase contrast microscope (Scale bar - 100 µM) or (X 148); Fig.2-b. Globular stage of somatic embryos under phase contrast microscope (Scale bar - 100 µM) or (X 148); Fig.2-c. Heart shaped stage under phase contrast microscope (Scale bar - 100 µM) or (X 148); Fig.2-d. Cotyledonary stage under phase contrast microscope (Scale bar - 100 µM) or (X 148).**

embryogenesis was induced at higher concentration of NOA. At higher levels of BAP (17.6 µM), TDZ (4.5 µM) and NOA (20 µM), the highest percentage of calli (26%) produced from leaf discs induced somatic embryos. These results indicated that PGRs combinations for induction of embryogenic calli in this cultivar are different as compared to other cultivars of grape viz. White Riesling, Thompson Seedless, Podarok, Margaracha, Krymskaya Zhemchuzhina, Seyval Blanc, Barbera, Nebbiolo, Appricot, Seyve Villard 5276, Dornfelder, Regent, Muller-Thurgau, Rupestris du Lot, Regale and Fry, which may be due to genome specificity of this cultivar.

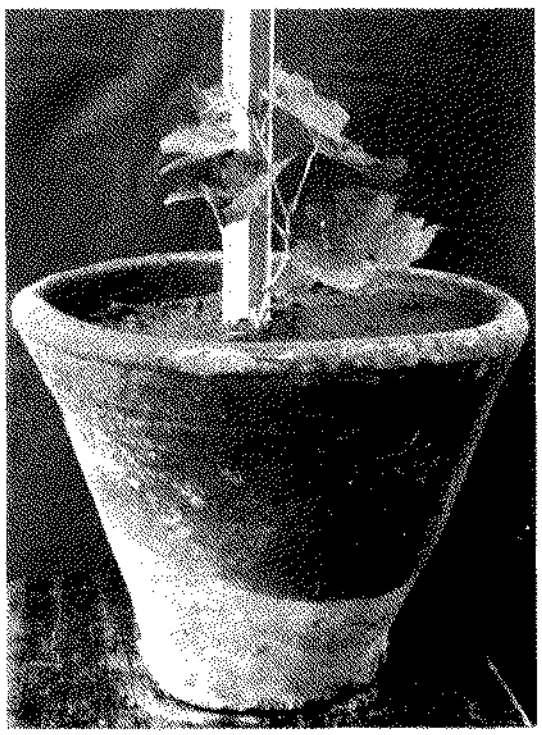
Trautmann *et al.* (1997) reported that leaves of *Vitis* cultured on NN medium containing 1 µM BA and 5 µM 2, 4-D, produced callus which showed pro-embryonic masses after 5 months. Proembryonic mass produced various stages of embryo development after 30 days on MS medium. However, results of the present investigation on leaf discs of grape cv. Tas-A-Ganesh revealed that time required for callus induction was 15 days and when the callus was allowed to grow for further 15 days and transferred to differentiation medium within 30 days, all types of embryo differentiation occurred (Fig.1). Hence, less time is required for embryogenesis with above mentioned PGRS combinations in this cultivar. Somatic embryos obtained from leaf discs excised from cv. Seyve Villard 5276 (*Vitis* sp.) and from a Brazilian interspecific hybrid (Passos *et al.* 1999).

Bornhoff and Harst (2000) studied the establishment of embryo suspension cultures of grapevine (*Vitis* L.) and homogeneous suspension cultures were maintained by fragmentation of embryos according to their developmental stage. Embryos of globular and heart stage (0.5 – 2.0 mm) were suitable for cultivation in suspension culture whereas further developed embryos (torpedo stage, 72.0 mm) were not suitable for cultivation in suspension. After transfer to a Linsmaier and Skoog (LS) medium the seedlings of Dornfelder and Muller-Thurgau regenerated to rooted plantlets *in vitro*. These plantlets were acclimatized and transferred to field conditions.

Leaf discs of four genotypes of grape (*V. vinifera* L.) when cultured on MS basal medium supplemented with 1mg BAP $l^{-1}$ and 0.1 mg 2,4-D $l^{-1}$, for 2 weeks under



3a



3b

**Fig.3-a. Germinated somatic embryo on hormone free medium; Fig.3-b. Hardened plantlet.**

dark conditions, produced calli. These subsequently differentiated into pro-embryos and embryos only if kept under conditions of low intensity (15 μE $m^{-2}$ $s^{-1}$) for 2 weeks before being transferred to conditions of high light intensity (60 μE $m^{-2}$ $s^{-1}$) (Das *et al.* 2002).

The plantlets of Pusa seedless, Beauty seedless, Perlett and Nasik were acclimatized in vermiculite and later transferred to soil in a green house (Das *et al.* 2002). *In vitro* grown emblings of cv. Tas-A-Ganesh were hardened in the soilrite in humidity chamber (80-85% RH) for 15 days and then in the green house for further 15 days. These were then transferred to field conditions. 50% emblings survived and maintained good growth (Fig. 3b).

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