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Standardization of Rapid *In Vitro* Regeneration Technique in *Vitis vinifera* cv. Manjari Naveen using Lateral Bud

S.D. Ramteke*, A.R. Langote, A.M. Dhole, A.H. Gavali and S.M. Khalate
ICAR-National Research Centre for Grapes, Pune-412307, Maharashtra, India
**Corresponding Author Email : sdramteke@yahoo.com*

Abstract

In vitro propagation is a commercial technology of tissue culture used in this era for plant regeneration around world. In the present investigation efficacious, rapid *in vitro* protocol for plant regeneration was developed using lateral buds in *Vitis vinifera* cv. Manjari Naveen on Murashige and Skoog (MS) medium amended by cytokinins and auxins. 0.1 % $HgCl_2$ was used to sterilize explants for 10 min and ascorbic acid (0.1 g/L) plus citric acid (0.15 mg/l) were used for minimization of browning in medium. Sterilized explants were cultured on MS media containing different concentration of BAP (0.1, 0.3, 0.5, 1.0 mg/ml and control) for shoot induction. The 0.5 mg/l BAP results in maximum shoot length (3.40 cm), high number of shoots (1.96) and low percentage of contamination for initiation. Afterwards, multiplication was obtained with the use of different concentration of cytokinins and auxins i.e., 0.1 mg/l BAP + 0.1 mg/l IBA, 0.5 mg/l BAP + 0.1 mg/l IBA, 1.0 mg/l BAP + 0.1 mg/l IBA, 1.5 mg/l BAP + 0.1 mg/L IBA. Control treatment was maintained without growth hormones. Among these, the maximum shoots multiplication was obtained on MS medium containing 0.5 mg/l BAP + 0.1 mg/l IBA. So, the protocol using MS medium containing combination of 0.5 mg/l BAP+0.1 mg/l IBA was standardized and used successfully for grapevine propagation. Therefore, use of auxins and cytokinins @ 0.5 mg/l BAP for shoot initiation, 0.5 mg/l BAP + 0.1 mg/l IBA for multiplication and 2.5 mg/L IAA for rooting can be exploited for *in vitro* propagation at commercial level in grapes.

Key words : *Vitis vinifera, lateral bud, cytokinins, auxins, in vitro, explants*

Introduction

Grapevine *(Vitis vinifera* L.*)* is an economically important deciduous woody fruit vine grown under tropical and subtropical regions of India (1). Grape berries are nutritionally very rich source of sugars and secondary metabolites such as polyphenols, anthocyanins, tannins, alkaloids, flavonoids, and other related compounds. Because of its high nutritional value grape berries are consumed freshly, dried or used for wine production from ancient times in Indian continent. Due to its high economical value special efforts are being adopted for surety of yield and to reduce yield losses due to diseases (2). Grapes were propagated conventionally through hardwood cutting, grafting, stem cuttings and layering. All these methods were time consuming and has extended juvenility period so require four to five years to establish successful grafts. Thus it was observed that grapevine production was hampered by its juvenility (1). Apart from that, conventional hybridization methods do not appear to be empirical for genetic improvement of classic cultivars to get high quality table grape varieties and good quality of wine. Grapevines are planted in the nursery during rainy season and one season's growth gives plants for transplanting to the vineyard. Furthermore, fungal diseases, insect pest attack hamper the propagation process which is season bound and slower process (3). However non-conventional propagation methods, *viz. in vitro* propagation methods of grapevine has emerged as a powerful tool for mass propagation and producing disease-free planting material under aseptic condition. Furthermore, it produces large number of plantlets in all the season in short time with high genetic and phytosanitary quality seasons (4). Micro-propagation method allows the rapid regeneration and mass propagation of any valuable genotype through organogenesis and embryogenesis (5, 6). Explants like apical meristems, axillary bud micro-cuttings or adventitious bud could be used successfully for grapevine propagation (6, 7, 8). Tissue culture techniques have been playing central role in germplasm conservation, embryo rescue, haploid or triploid production, hybridization and cybridization (9). Similarly, micro-propagation is practical efficient way to propagate woody plants like grapes but for better success it should be combined with shoot multiplication, rooting, and hardening (10, 11). So, the purpose of present investigation is to standardized protocol for multiple shoot regeneration from lateral bud through tissue culture.

Materials and Methods

The experiment was carried out at ICAR-National Research Centre for Grapes, Manjari Farm, Pune, during 2020. Lateral bud cutting having two to three buds each were collected from six to seven years old vines of Manjari Naveen from experimental vineyard of ICAR-National

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Table-1 : Effect of different concentration of BAP on shoot initiation.

BAP Concentration (mg/l)	Sprouting (%)	Contamination (%)	No. of Shoots /Explants	Shoot length (cm)	Leaf numbers
0.0	10%	30%	0.25	0.20	0
0.1	50%	20%	1.0	1.48	3
0.3	75%	50%	2.0	2.10	4
0.5	100%	10%	4.0	3.40	8
1.0	85%	60%	1.0	2.39	4
SE(m)	0.91	0.69	0.02	0.03	0.05
CD @ 5%	2.66	2.01	0.07	0.08	0.15
CV%	3.19	4.53	3.34	3.05	3.05

Table-2 : Effect of plant growth regulators on shoot multiplication.

Growth regulator combination	No. of shoots produced	Shoot length (cm)
Control	1.0	0.17
0.1 mg/l BAP+0.1 mg/l IBA	2.0	0.50
0.5 mg/l BAP+0.1 mg/l IBA	3.0	1.0
1.0 mg/l BAP+0.1 mg/l IBA	2.0	0.25
1.5 mg/l BAP+0.1 mg/L IBA	Callus	Callus
SE(m)	0.04	0.01
CD @ 5%	0.11	0.04
CV%	5.06	7.48

Table-3 : Effect of Auxins on rooting of grapevine cv. Manjari Naveen.

IAA Concentration (mg/L)	Percentage of rooting	Number of roots per shoot
0.0	13.332	1.2
0.5	26.664	1.4
1.0	33.33	1.8
1.5	39.996	3
2.0	46.662	3
2.5	59.994	4.2
SE(m)	9.19	0.40
CD @ 5%	NS	1.15

Research Centre for Grapes, Pune, India during the year 2020. The vineyard is situated at mid-west of Maharashtra state at an altitude of 559 m (18.32° N and 73.51° E) above the sea level with tropical wet and dry climate in which the average temperature ranging between 25-35° C during the peak period of season.

Explant Preparation : Explants placed in moist paper towels in the laboratory toavoid desiccation of explants. The explants (1- 2 cm), lateral-bud cuttings are prepared by removing all the extraneous tendrils and leaves and the lateral bud cuttings with two buds are prepared.

Media sterilization : Murashige and Skoog (1962) (MS) medium supplemented with 0.5 mg/L BAP, sucrose 30 g/L as carbon source, ascorbic acid (0.1 g/L) and citric acid (0.15 mg/l) was added for browning problem. Medium pH was adjusted to 5.8 and agar (7gm/l) was added as a solidifying the medium; 15ml of medium was poured into each test-tube (15 cm length, 2.5 cm dia.), and the medium was sterilized in an autoclave at 121°C, 15 psi pressure for 20 min.

Surface-sterilization and inoculation of the explants : As a preliminary treatment, the explants were thoroughly washed under running tap water three times to remove all dust and debris adhered to its surface, and washed with detergent for 10 min. Sterilization was carried out aseptically under a laminar air-flow cabinet, which was sterilized by UV light for 45 minutes and wiped with 95% alcohol for 15 minutes. Explants were treated with 'Tween-20' surfactant solution (1 to 2 drops in 100 ml distilled water), followed by pre-soaking in a broad-spectrum systemic Fungicide, Bavistin @ 250 mg/l for 15 min and surface-sterilized with 0.1% $HgCl_2$ for 10 min, followed by three rinses in sterile distilled-water. Then sterilized MS medium amended with 0.1, 0.3, 0.5 and 1mg/l BAP was used for explants inoculation. Each treatment was replicated five times and medium without BAP was maintained as control. The tubes were incubated in a growth room facilitated with 3000 Lux light intensity under a photoperiod of 16 h light/ 8 h dark, and maintained at a temperature of 25±2 ºC.

Shoot multiplication : After 2 weeks of initiation, the initiated culture (0.5 mg /l BAP) shifted to a medium that was for shoot multiplication. Shoots were cut and

transferred to autoclaved media containing 0.1 mg/l BAP, 0.5 mg/l BAP, 1.0 mg/l BAP and 1.5 mg/l BAP in combination with 0.1 mg/l IBA in laminar air flow and pH was adjusted to 5.8. The test tubes were sealed with parafilm and incubated at 1000-3000 Lux light intensity under a photoperiod of 16 h light /8 h dark, and maintained at a temperature of 25 ± 2°C. Special care of inoculation room, growth conditions were taken to minimize the contamination. Culture bottles were transferred to growth room for two weeks.

Shoot rooting : Individual propagated shootlets (3- 4 cm) were separated and cultured in the root induction medium supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L IAA. The percentage of rooting and average root numbers (roots/shoot) were observed and recorded after two weeks.

Hardening of plantlets : After three weeks of growth of rooted plants in culture bottles the rooted plantlets were washed carefully and treated with broad-spectrum systemic fungicide, Bavistin @ 250 mg/l for 15 min. Then rooted plantlets were potted into plastic cup containing peat moss and perlite (1:2 v/v), covered with transparent plastic sheet to maintain optimum humidity. Then plants were acclimatized at greenhouse conditions with 25±1 °C temperature and 75–85% relative humidity. After acclimatized for three weeks plastic sheets were removed and maintained for further growth.

Results and Discussion

Surface-sterilization and inoculation of the explants **:** In the present investigation explants were derived from field-grown grapevines were carefully surface sterilization because it is usually considered to be a serious problem. The experimental result of this study revealed that 1 to 2 drops of tween 20 in 100 ml water and 250 mg Bavistin for 15 min and 0.1% $HgCl_2$ for 10 min was effective for sterilization of explants since only less than 2% contaminated explants were found. Furthermore, the use of ascorbic acid (0.1 g/L) and citric acid (0.15 mg/l) in medium found quite effective to control the browning of media since, there was no browning observed in any tubes.

Shoot initiation : Result on the effect of BAP on shoot initiation was showed that shoots were induced in all treatments including control. The survival % of shoot showed significant variation of different concentration of BAP. In present study the maximum percentage of survival (99%) and shoot length (3.40 cm) was achieved with 0.5 mg/l BAP concentration (Fig.-1). The maximum shoot length (3.40 cm), no of shoots (1.96), and leaf no (8) were recorded in 0.5 mg/l BAP media. The high concentration of BAP (>0.5 mg/l) gives hyperhydricity in explants. At higher concentration levels of BAP growth of axillary bud and shoot multiplication were increased but hyperhydricity was also resulted. Result showed that 0.5 mg/l BAP get best results for shoot initiation, i.e. maximum shoot length, maximum sprouting percentage, lower

Fig.-1 : Growth of lateral bud on MS medium containing 0.5 mg/l BAP.

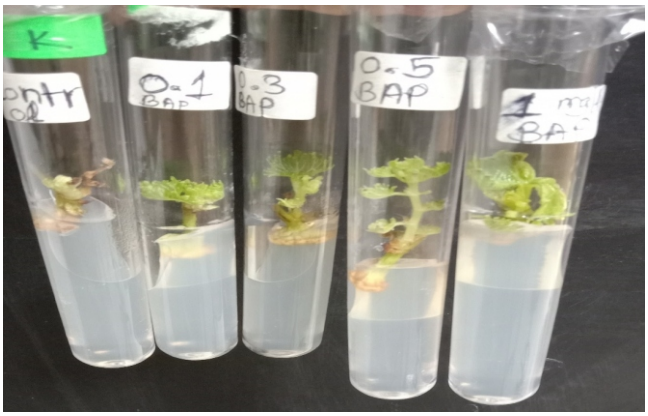

Fig.-2 : Effect of different concentration of BAP on shoot multiplication.

Fig.-3 : Rooting on medium containing 2.5 mg/L IAA.

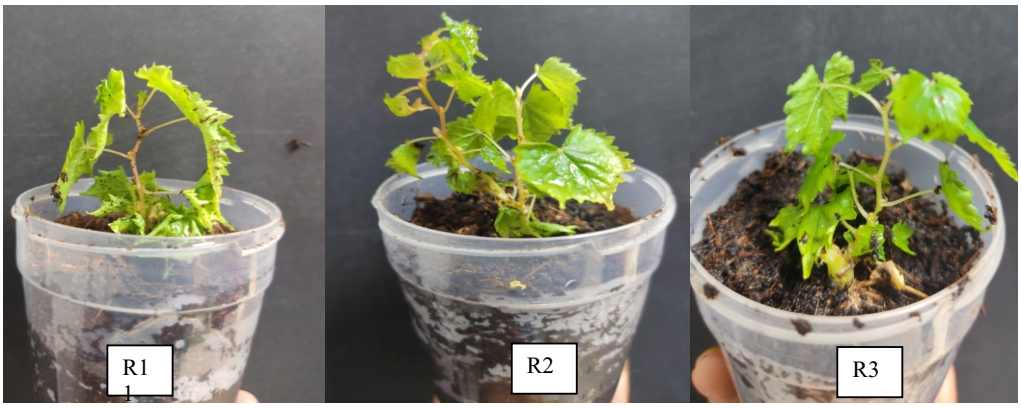

Fig.-4 : Hardening of healthy plants from shoot induction BAP @ 0.5 mg/l, shoot multiplication with 0.1 mg/l IBA in combination with 0.5 mg/l BAP and rooting with 2.5 mg/L IAA.

contamination %, maximum no of shoots per explants (Table-1 and Fig.-1).

Shoot multiplication : After 3-4 weeks the explants were transfered into the multiplication media for comparative study of multiplication stage there three different media used i.e. 0.1 mg/l BAP, 0.5 mg/l BAP, 1.0 mg/l BAP and 1.5 mg/l BAP in combination with 0.1 mg/l IBA. It was found that the 0.5 mg/l BAP in combination with 0.1 mg/l IBA resulted in highest shoot multiplication and gave better result after three weeks of inoculation on multiplication media (Table-2 and Fig.-2). The medium containing 0.5 mg/l BAP +0.1mg/l IBA increased the number and length of shoots after 10 DAI.

***In vitro* rooting :** Shoot obtained from multiplication stage were transferred to medium supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L IAA and found successful rooting in all transferred plants. The highest rooting percentage (59.99) and root numbers per shoot (4.2) was observed in medium containing 2.5 mg/l IAA (Fig.-3). Roots were visible through plastic bottle after 15 days of inoculation.

Hardening of plantlets : *In vitro* rooted plants were hardened successfully on coco peat in controlled conditions (Fig.-4).

Woody plants like grapevine require one or two years to establish in field through conventional propagation methods like hard wood cutting or grafting. Present investigation showed that the healthy and uniform plantlets can be obtained through tissue culture technique within two- three months. According to (1) sterilization of lateral bud is a serious problem in all woody plants as explants were derived from field. In most of studies lateral buds were selected either from *in vitro* grown plants or from plants grown in the greenhouse. Oxidative browning was also a problem in woody plants like grapes; so in present study browning problem was minimized significantly with the use of ascorbic acid and citric acid.

It is well documented that the BAP is the most effective among other cytokinins for inducing shoot development of lateral bud in *vitis* (12). Previously, (13) reported that the BAP @ 0.5 mg/l gives morphologically best shoots induction. Hyperhydricity has been previously reported in grapevine tissue culture (12) and considered a serious problem because vitrificated shoots are not micro-propagated further. Therefore, in present study the concentration of BAP to 0.5 mg/l was standardized to obtain optimum shoot number, shoot length, leaf numbers and to avoid the hyperhydricity due to higher concentrations of BAP.

Additionally, IBA in combination with BAP resulted in better shoot initiation (14) and maximum mean number of shoots were observed in the media with combination of 1.0 mg/l BAP +0.1 mg/L IBA in *Vitis vinifera* cv. Chenin Blanc (13). It is also reported that 1.5 mg/l BAP +0.5 mg/l NAA showed starting to formation of callus and 1.0 mg/l BAP +0.1 mg/l GA3 gives 60% results in shoot initiation.(14) reported that the medium containing 1.0 mg/l BAP +0.1 mg/l GA3 gives highest shoot number per explants. Furthermore, (4) reported that the highest number of new micro shoots was observed in culture supplemented with 0.6 mg/l BAP with combination of 0.2 mg/l KIN and 0.5 mg/l GA3. Combination of GA3 with BAP and KIN stimulate elongation of shoots.

Conclusions

In present investigation, use of different PGRs for *in vitro* shoot induction from lateral bud in *Vitis vinifera* cv. Manjri Naveen was reported first time. Since according to the literature surveyed, *in vitro* propagation method is best than conventional propagation. It is standardized that the surface sterilization with 0.1% $HgCl_2$ is best for lateral bud in grapevine. The protocol for *in vitro* shoot induction of lateral bud was standardized with the use of BAP @ 0.5 mg/l, shoot multiplication with 0.1 mg/l IBA in combination with 1.0 mg/l BAP and rooting with 2.5 mg/L IAA. By using this protocol hardened plantlets could be produced successfully within two months. Thus, present

standardized protocol could be helpful for the commercial production of planting material of Manjari Naveen grapevines.

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