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A one step *in vitro* cloning procedure for Red Globe grape: The influence of basal media and plant growth regulators

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Abstract

Earlier studies have shown that the degree of success at each stage of micropropagation in grapevine is genotype dependent; hence it becomes imperative to optimize culture conditions for rapid propagation of a variety. Present report describes two approaches of *in vitro* propagation of a *Vitis vinifera* cultivar, Red Globe. In one approach, whole plants could be developed from single node segments by bud break and direct rooting *in vitro*. Eight different basal media tried showed different morphogenetic responses**.** In second approach, multiple shoots were induced in nodal segments cultured on MS basal medium supplemented with BA (8.88 µM). Also, second crop of shoots could be induced in left over nodal segments devoid of shoots. Rooting of shoots could be induced *in vitro,* both in semi-solid or liquid media and also *ex vitro* by pulse treatment of IAA (2.85 µM) + NAA (2.70 µM). Plant establishment in later case was 80%. A simple procedure described here can complement conventional methods, currently being used in propagation of this important grape variety.

Key words: Auxin pulse, benzyladenine, grape, micropropagation, Red Globe, *Vitis vinifera*

Introduction

Due to heterozygous nature grape varieties are mostly propagated by vegetative means. Application of plant tissue culture techniques in propagation and improvement of grapevines has been reviewed by several workers (Krul and Mowbray, 1984; Gray and Meredith, 1992; Torregrosa *et al.*, 2001). The technique has been used to propagate pathogen free grapevine stock (Duran-Vila *et al.*, 1988). Micropropagation complements the conventional technique when a large number of propagules of a particular variety are required in a shorter time. Earlier studies on *in vitro* propagation of *Vitis* have indicated that the degree of success at each stage of culture is genotype dependent and varies under a given set of culture conditions (Barlass and Skene, 1980; Monette, 1988; Botti *et al.*, 1993). Hence, it becomes essential to optimize culture conditions for a particular clone / cultivar / rootstock or newly bred line that needs large scale planting but availability of sufficient planting stock is a limitation. The present communication describes influence of eight basal media and growth regulators on micropropagation of Red Globe, a *Vitis vinifera* cultivar. The variety is in great demand due to its attractive reddish-purple colour; taste bud arousing flavour and appealing large plum-size berries with uniform bunches.

Material and methods

Twigs of field grown, disease free vines of Red Globe were collected from the vineyard of National Research Centre for Grapes, Pune. These were defoliated and cut into single node segments (2 cm). The explants were dipped in 1% Labolene solution for 10 min; rinsed with tap water; submerged in 0.1% Bavistin solution; kept on a shaker (120 rpm) for 2 h and thereafter rinsed three times with sterile water in a laminar flow hood. These were then disinfected with 0.1% mercuric chloride solution for 10 min and rinsed three times with sterile water. The explants were finally blotted dry on sterile filter paper and inoculated on medium in glass test tubes (150 X 25 mm).

For budbreak, eight different basal media – MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980), NN (Nitsch and Nitsch, 1969), B5 (Gamborg *et al.*, 1968), ER (Eriksson, 1965), LS (Linsmaier and Skoog, 1965), C_2d (Chee and Pool, 1987) and GNMG (Galzy *et al.*, 1990) devoid of growth regulators were tested. Another experiment with MS medium and range of BA concentrations (0.04 to 11.1 µM) was undertaken to maximize budbreak. To obtain second crop of shoots, primary nodal segments left after excising the grown axillary shoot (hereinafter referred to as mother explant) instead of its discard, were transferred to WPM or MS with or without BA (4.44 and 8.88 µM).

For induction of multiple shoots, axillary shoots obtained from primary nodal segments were inoculated (S0) in test tubes having MS medium with BA (2.22 to 8.88 µM). After 30 d, explants showing multiple shoots were transferred to fresh medium in glass bottles. This was continued at an interval of 30 d until five transfers (from S1 to S5). To test the effect of inoculum's density per culture vessel, two, three, four or five shoot clumps per culture bottle were inoculated on MS with BA at 4.44 or 8.88 µM. Two sets of experiments were carried out for elongation of *in vitro* shoots. In the first set, shoots less than 3 cm in length were inoculated on WPM supplemented with or without BA (2.22-8.88 µM). In the second set, multiple shoot clumps with shoots of <1.5 cm in length were kept for elongation. These were inoculated in glass bottles containing MS supplemented with BA (2.22 or 4.44 µM) and NAA (0.54 µM).

For *in vitro* rooting, shoots more than 3 cm were inoculated in test tubes containing half or full strength MS or WPM supplemented

with NAA (0.54 - 1.07 µM) or IAA (0.57 - 1.14 µM) or IBA (0.49 - 0.98 µM) or IPA (0.53 - 1.06 µM) with or without agar. Liquid media had filter paper bridges. *In vitro* raised shoots, more than 7 cm in length were given pulse treatment of different auxins, IAA (2.85 - 5.71 µM) or IBA (2.46 - 4.90 µM) or IPA (2.64 - 5.29 µM) or NAA (2.69 - 5.37 µM) either singly or in combination for 10 min and then planted in plastic cups containing a mixture of coco-peat + soil + sand (1:1:1). Untreated shoots served as control. Shoots rooted were taken out of the culture tubes, their roots gently washed with water to remove adhering medium and were transferred to plastic cups containing the above mentioned mixture. Plants were acclimatized by the Sachet technique (Ravindra and Thomas, 1985). Plants after transfer to cups were kept in continuous light of 24.4 µmol m^{-2} s^{-1} at 25 ± 2^0C. Thereafter, these were shifted to another growth room at ambient temperature (35 ± 2^0C). Establishment of plants was recorded after 30 d.

All the media were supplemented with sucrose 20 g L^{-1} and gelled with agar 7 g L^{-1}. The pH of the media was adjusted to 5.8 before autoclaving at 121^{0}C for 20 min. Cultures were incubated under 16 h photoperiod obtained with cool light fluorescent tubes with light intensity of 24.4 µmol m^{-2} s^{-1} at 25 ± 2^0C. Experiments were repeated at least three times. Observations were recorded at monthly interval. The experiments were conducted in Completely Randomized Design and the results were subjected to analysis of variance.

Results and discussion

Bud break in nodal segments commenced from the fifth day of inoculation and continued up to 20th day and thereafter shoots put forth rapid growth. Among the eight basal media tested, C_2d, LS and WPM without any growth regulators induced 92, 90 and 84% budbreak, respectively. Induction of two or more shoots in maximum explants (76%) was observed in C_2d medium. NN induced the minimum response (Table 1). In addition to budbreak, nodal explants both in WPM and B5 induced rooting at the base in 70% of explants. In case of NN, it was 46%. In all other media rooting was very low. These rooted nodal segments with primary shoots could be established on potting and were hardened by the Sachet technique. Thus, no special difficulty was faced with nodal culture producing entire plantlet.

Eight different nutrient media induced different morphogenetic responses in nodal segments. Shoots in C_2d were found to be stunted, succulent with light green, thick leaves and glossy in appearance. Basal media LS and Eriksson showed necrosis in shoot tip, which continued to the entire shoot and caused drying of the whole shoot. Shoots in NN lacked vigour, had thin stems with dark green leaves. MS resulted into comparatively better shoots with normal internode and light green leaves. Also, shoots on MS were most vigourous as compared to other media tested. The shoots in B5 were similar to those observed in MS except that the internode was slightly thicker. The shoots in WPM lacked vigour and had thin, lanky stems showing twining habit with thin foliage. Of the eight media tested, MS was found to be the most suitable medium resulting into vigorous shoots. Hence, for multiple shoot induction experiment, only MS was used.

In a similar study on basal medium, Reisch (1986) observed significant differences in growth in grape cultivar White Riesling with MS half and MS full medium. However, in contrast to the present study, Gray and Benton (1991) observed stunted growth in shoots of Muscadine grape cultivars when WPM was used. Genotypic variability within *Vitis vinifera* cultivars cultured *in vitro* has earlier been reported (Harris and Stevenson, 1982; Chee and Pool, 1983; Galzy *et al.*, 1990). Varying response of different genotypes to different basal media could be due to variations in nutrient compositions. For example, amount of $CaCl_2$ is higher in MS, LS and Eriksson as compared to WPM and NN, while in C_2d and GNMG, it is substituted by $Ca(NO_3)_2$. Similarly, Potassium Iodide (KI) is absent in WPM, NN, C_2d and Eriksson while it is present in GNMG, B5, LS and MS though in different quantities. Also amounts of $MnSO_4$ vary in the eight basal media tested.

Galzy (1969) demonstrated that mineral requirement varied with the morphogenic process: strong K and N concentrations proved favorable to shoot development but impeded root growth. Chee and Pool (1987) working with grape tissues have reported that lower concentrations of KI and $MnSO_4$ in the medium were good for maximum shoot production and incorporation of $Ca(NO_3)_2$ instead of $CaCl_2$ produced shoots of good quality. Present study corroborates these findings since maximum results of budbreak of shoots were obtained from explants inoculated on C_2d though shoots obtained from explants inoculated on MS medium were comparatively healthy and vigorous. Besides nutrients, differences in *in vitro* response between genotypes of different species may be related to differences in endogenous

Table 1. Effects of eight basal media on morphogenesis in nodal segments of *Vitis vinifera* cv. Red Globe

Basal medium	Explants showing bud-break* (%)	Explants showing single shoots (%)	Explants showing 2 or more shoots(%)	Total number of shoots obtained	Soots elongated (%)	Average shoot height (cm) ± S.D.	Explants showing rooting at the base (%)
NN	42	38	04	23	82.6	2.13 ± 0.10	46
C_2d	92	16	76	97	44.0	3.52 ± 0.46	02
B5	70	32	38	58	52.0	3.90 ± 0.23	70
MS	80	18	62	96	37.0	1.86 ± 0.35	10
LS	90	24	66	98	38.0	1.82 ± 0.36	14
ER	68	04	64	83	39.0	1.19 ± 0.10	04
WPM	84	40	44	68	44.0	4.25 ± 0.50	70
GNMG	62	56	06	25	96.0	1.49 ± 0.18	00
LSD (*P*=0.01)						0.53	

* Based on 50 explants, ± SD = Standard deviation

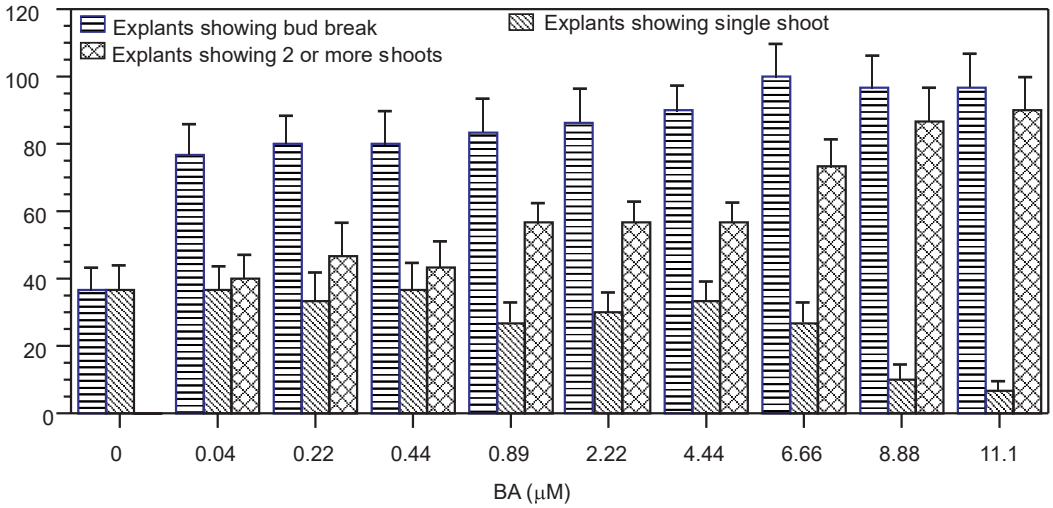

Fig. 1. Effects of BA concentration on budbreak and number of shoots in nodal segments of Red Globe following 30 d of culture, basal medium – Murashige and Skoog, (1962); bars indicate standard error.

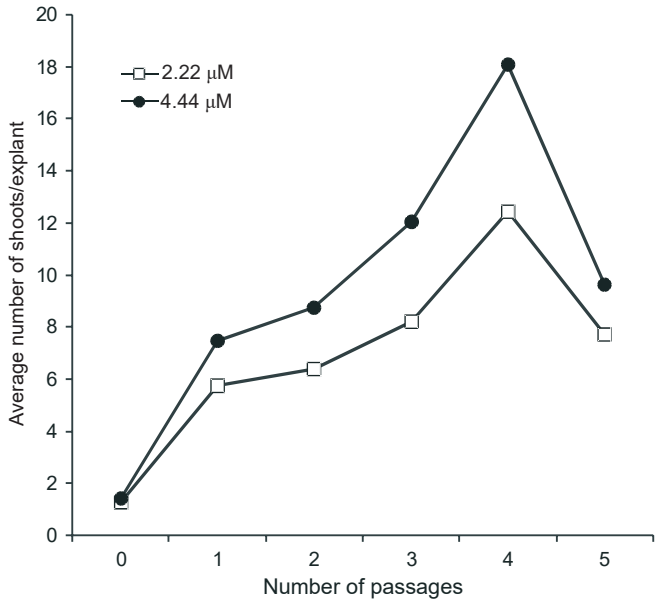

Fig. 2A. Effects of BA concentration and number of passages on shoot proliferation in Red Globe grape.

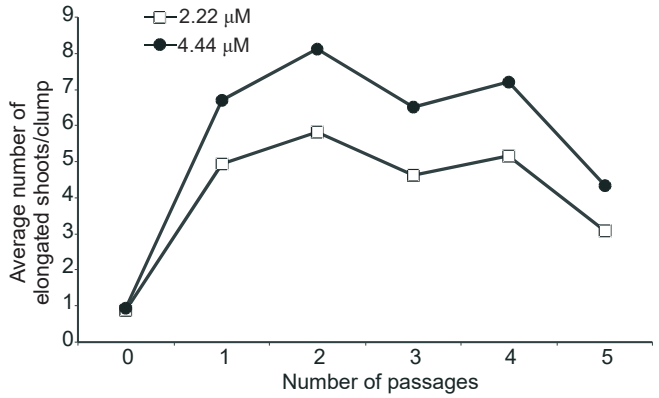

Fig. 2B. Effect of BA concentration and number of passages on elongation of shoots in Red Globe grape.

levels of phytohormones (Looney *et al.*, 1988; Alvarez *et al.*, 1989; Gronroos *et al.*, 1989).

In the second experiment, BA at 6.66, 8.88 and 11.1 μM resulted into 100, 96.66, and 96.66% of explants showing budbreak. There was marginal difference in the response of BA levels from 0.04 to 0.89 μM. However, a linear increase in number of two or more shoots per explant was observed on increase in BA concentration from 0.04 to 11.1 μM. Maximum response (90%) of two or more shoots per explant was recorded with BA at 11.1 μM (Fig. 1). Addition of BA in MS not only induced bud break in higher number of nodal explants but shoots were of better quality in terms of vigour and leaf colour. Positive influence of BA in establishment of axenic shoots in grapes has earlier been documented in several reports (Chee and Pool, 1983; Reisch, 1986; Lee and Wetzstein, 1990; Robacker and Chang, 1992; Torregrosa and Bouquet, 1995; Mhatre *et al.*, 2000). A second crop of shoots could be induced in mother explants cultured on WPM or MS with BA at 4.44 or 8.88 μM. The maximum shoot induction was obtained in MS with BA (8.88 μM) considering both single and two or more shoots in explants (data not shown).

Primary shoot used as explant, induced maximum multiple shoots (2.27) per explant on an average in MS supplemented with BA (8.88 μM) after 30 days of inoculation (S0). Though marginally higher, a linear increase in number of shoots was observed on increase in BA concentration from 2.22 to 8.88 μM though reverse was true for number of shoots elongated per explant. Medium without BA (served as control) showed the least number of shoots as well as least number of elongated shoots per explant. On transfer of these shoots to fresh media (S1) in glass bottles, number of multiple shoots increased several fold and showed linear increase with increase in BA concentration (Fig. 2A). The same trend was observed with number of elongated shoots per explant (Fig. 2B).

It was observed that BA concentrations at 6.66 and 8.88 μM showed higher number of shoots and elongated shoots per explant from subcultures S0 to S1 however, shoots produced were hyperhydric and showed abnormalities in leaf shape. The leaves were dark green with glossy appearance. Also, shoots

Table 2. Effect of basal media and BA on shoot elongation in cv. Red Globe

Basal medium + BA (µM)	Percent of shoots elongated*	Average shoot length (cm) ± SD
WPM + BA (4.44)	66.67	7.50 ± 0.45
WPM + BA (8.88)	53.33	6.38 ± 0.45
WPM	13.33	1.50 ± 0.17
MS + BA (4.44)	93.33	6.75 ± 0.26
MS + BA (8.88)	61.67	4.50 ± 0.30
MS	30.00	1.00 ± 0.06
LSD (*P*=0.01)		0.56

* Based on 60 explants, ± SD = Standard deviation

were short and compact in the form of clumps. Hence for further subcultures, these two BA concentrations were discontinued. On repeated sub-cultures of these shoot clumps from S1 to S4 at an interval of 30 d, a drastic and linear increase in number of shoots was observed (Fig. 2A). As observed earlier this could be due to axillary branching in shoots clusters or occurrence of adventitious organogenesis (Chee and Pool, 1985). However, in S5, number of shoots per clump decreased drastically indicating toxicity.

It was found that the density of clumps inoculated per culture vessel influenced the rate of shoot proliferation for all the BA levels. An inverse correlation was observed between BA concentration and density of clumps. Higher the BA concentration more the number of shoots per clump while reverse was true with density *i.e.* higher the number of clumps per vessel lesser the number of shoots per clump. The maximum number of average shoots (15.4) per clump was obtained with two clumps per culture vessel in medium containing BA 8.88 µM (data not shown).

Since all the shoots in multiple shoot clumps did not elongate, it was necessary to carry out a separate experiment for elongation of shoots. In one experiment, shoot clump was kept as explant on two media containing MS as basal medium supplemented with NAA at 0.54 µM and BA at two concentrations 2.22 and 4.44 µM. At both the BA concentrations, there was very marginal difference in average number of shoots elongated 12.95, 13.63 with average shoot length of 5.90 and 5.33 cm, respectively. Inoculation of shoot clumps for elongation of shoots has advantage since several shoots elongated simultaneously instead of one, saving time and labour.

In the other experiment, single shoots (2-3 cm) isolated from multiple shoot clumps were kept on WPM and MS basal media without BA or with BA at 4.44 and 8.88 µM. Maximum percentage of shoots (93.33%) elongated on MS with BA (4.44 µM) giving an average shoot length of 6.75 cm followed by WPM (66.67 %) giving an average shoot elongation of 7.50 cm. BA concentrations lower than 4.44 µM showed bolting of shoots while BA at higher than 8.88 µM resulted into thick, stunted, succulent and hyperhydric shoots (data not shown). This is in conformity with observations recorded in three grapevine cultivars by Mhatre *et al.* (2000). Basal medium without growth regulators showed shoot necrosis and was not effective in shoot elongation.

Rooting of *in vitro* shoots is influenced by several factors, of which growth regulator requirement is of major importance. Though rooting of *in vitro* raised shoots could be induced in MS half or full strength basal media (agar solidified or liquid)

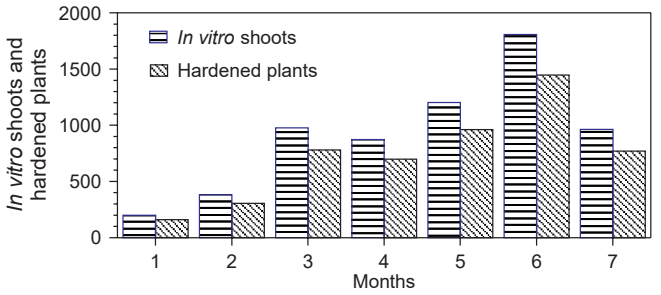

Fig. 3. Number of *in vitro* shoots and hardened plants obtained in a span of seven months (starting with 100 nodal segments).

supplemented with or without NAA (0.54 - 1.07 µM) or IAA (0.57 - 1.14 µM) or IBA (0.49 - 0.98 µM) or IPA (0.53 -1.06 µM) however, quality of roots was better on incorporation of NAA at 0.54 - 1.07 µM in the medium. Number of days required for rooting was less for the shoots inoculated in the liquid medium as compared to the solidified medium. In MS half or MS full medium devoid of growth regulators, the quality of roots was poor and shoots lacked vigour. Addition of NAA in the rooting medium induced longer roots with primary and secondary branching. This was reflected in the higher survival of rooted shoots (83%) when treated with NAA (data not shown). In earlier reports on grapevine, it was documented that auxin stimulated root initiation but inhibited subsequent root growth (Galzy, 1969), and that its appropriate concentration was of critical importance. In previous studies, it was observed that effects of auxins on rooting depend on mineral composition of the nutrient media (Novak and Juvova, 1983; Zlenko *et al.*, 1995). Root initiation was not influenced by salt concentration, but root growth was enhanced when salt concentration of rooting media was reduced (Harris and Stevenson, 1979).

Ex vitro auxin pulse treatment of *in vitro* shoots for 10 min induced direct roots. Shoots given a pulse treatment with auxin mixture of IAA (2.85 µM) and NAA (2.70 µM) showed 80% plant establishment. Pulse treatment of IAA (5.7 µM) or NAA (5.4 µM) alone gave rise 73 and 70% establishment, respectively. A mixture of IAA (5.7 µM) and NAA (5.4 µM) resulted into lower percent (53%) of establishment. Shoots directly transferred to potting mixture without any auxin pulse did not induce roots and could not establish. Plants could be acclimatized by the sachet technique which is simple, effective and does not require any sophisticated set-up. It was found that shoots planted in a mixture of coco-peat + soil + sand (1:1:1) showed a plant survival of 75%.

Thus, present communication describes two routes of micropropagation in grapevine cultivar Red Globe. In one route, whole plants could be developed from single node segments by bud break and direct rooting. To our knowledge no such systematic study on basal media has been reported so far for tissue culture of grapevines. In second route, larger number of plants could be obtained by multiple shoot induction, shoot proliferation and *ex vitro* rooting by auxin pulse treatment. Within seven months period, about 100 single node segments could give rise to about 5442 *in vitro* shoots and 4354 established plants compared to conventional vegetative cutting method where each three to five node cutting yields only one plant (Fig. 3). Tissue culture plants produced, have been supplied to National

Research Center for Grapes (NRCG), for its performance in the field. A simple *in vitro* propagation procedure described here can complement conventional methods, currently being used in propagation of this important grapevine variety.

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