RESEARCH PAPER | Asian Journal of Bio Science, Volume **8** | Issue 1 | April, 2013 | 19-24
Received : 04.12.2012; **Revised :** 14.02.2013; **Accepted :** 18.02.2013

In vitro regeneration of grape (*Vitis vinifera* L.) cv. PERLETTE

MAHITAL JAMWAL, BARINDER SINGH, NIRMAL SHARMA, RAJESH KUMAR, ARTI SHARMA, R.M. SHARMA, V.K. WALI **AND** A.M. PARMAR

Division of Fruit Science, Sher-e- Kashmir University of Agricultural Sciences and Technology, Chatha, JAMMU (J&K) INDIA
Email : mahital_jamwal@rediffmail.com

Factors like slow and seasonal multiplication and infection with pathogens have constrained the use of conventional plant propagation methods, thus lead to development of new and novel methods of propagation like *in vitro* multiplication which ensures the production of virus and disease free elite planting material in large numbers. In the present work, *in vitro* regeneration protocol has been standardized for grape cv. Perlette which is conventionally propagated through hardwood cuttings. The highest explant establishment (86.66%) and lowest days (12.00) for explants establishment were obtained in MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l kinetin. Best shoot proliferation (3.33 shoots/culture) was obtained in MS medium fortified with 1.0 mg/l BAP and 0.5 mg/l kinetin. The media constituting MS + 1.0 mg/l NAA was best for rooting of *in vitro* raised shoots, yielding 73.33 per cent rooting with an average root length of 4.43 cm. The most suitable potting media for *in vitro* raised plantlet hardening of grape cv. Perlette constituted sand (1part) soil (1part) FYM (1part) vermiculture (1part) which resulted in 73.33 per cent plantlet survival.

Key words : Grape, *In vitro* regeneration, Tissue culture, *Vitis vinifera* L., Perlette

How to cite this paper : Jamwal, Mahital, Singh, Barinder, Sharma, Nirmal, Kumar, Rajesh, Sharma, Arti, Sharma, R.M., Wali, V.K. and Parmar, A.M. (2013). *In vitro* regeneration of grape (*Vitis vinifera* L.) cv. PERLETTE. *Asian J. Bio. Sci.,* **8** (1) : 19-24.

INTRODUCTION

The cultivated grape (*Vitis vinifera* L.) is a member of family Vitaceae and a hybrid of two American species *Vitis vulpine* and *Vitis labrusca*. It is well known for its delicacy and is a good source of minerals like calcium, phosphorus, iron and vitamins like B_1 and B_2. Grape is cultivated on an area of 7,197 thousand hectares worldwide with an annual production of about 68 million tones, Spain, France, Italy and USA being the leading grape producing countries in the world however, the average productivity is highest (25.80 t/ha) in India producing 2.26 million tones fresh grapes from an area of 87.7 thousand hectare (Anonymous, 2010).

Maharashtra, Tamil Nadu, Karnataka, Punjab and Andhra Pradesh are the major grape growing states of India (Anonymous, 2010). During the last two decades, grape cultivation has gained popularity among fruit growers of north India, especially Punjab, Haryana, Himachal Pradesh and Jammu and Kashmir States. The Perlette grape has monoculture in north India with a more than 90 per cent of total acreage under this variety (Bindra and Brar, 1996). In recent past, it has been observed that non-availability of adequate number of true to type, disease free planting material has been the major constraint for establishment of ideal vineyards. Grape has been vegetatively propagated through hardwood stem cuttings and may carry plant pathogens along with the plant material causing finally reduced vigour of plants. While preparing the cuttings, special care has to be taken to see that the vines are perfect and free from pests. Moreover, the rate of multiplication by conventional methods is rather slow and they do not ensure the production of virus and disease free elite plant material. However, rapid multiplication of newly introduced improved cultivars, exchange of virus-free plant materials, maintenance and conservation of genetic resources, the testing of *in vitro* shoots for resistance to diseases, insects and abiotic stresses are some of the advantages of *in vitro* propagation. *In vitro* plant regeneration of grapes in different parts of the world has shown encouraging results to produce large number of elite virus free plants round the year. Therefore, attempt has been made to standardize the *in vitro* regeneration protocol for leading north Indian grape (*Vitis vinifera* L.) cv. Perlette.

RESEARCH METHODOLOGY

In vitro regeneration studies in grape (*Vitis vinifera* L.) cv. Perlette were carried out in tissue culture laboratory located at Udheywalla campus of Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, J & K, India. In order to study the morphogenic response of grape explants, the most widely accepted MS medium (Murashige and Skoog, 1962) was used as basal medium prepared by mixing the required quantity of each of the five stock solutions in 500ml of distilled water by continuous stirring. Myo-inositol (100 mg/l) and 3 per cent sucrose, unless mentioned otherwise, were added to the solution. In order to standardize the most suitable culture establishment medium, fifteen treatments of growth regulators either singly or in combination, with MS basal media (T_1: MS Basal, T_2: MS + 0.5 mg/l BAP, T_3: MS + 1.0 mg/l BAP, T_4: MS + 2.0 mg/l BAP, T_5: MS + 4.0 mg/l BAP, T_6: MS + 1.0 mg/l kinetin, T_7: MS + 2.0 mg/l kinetin, T_8: MS + 0.5 mg/l BAP + 1.0 mg/l kinetin, T_9: MS + 1.0 mg/l BAP + 1.0 mg/l kinetin, T_{10}: MS + 2.0 mg/l BAP + 1.0 mg/l kinetin, T_{11}: MS + 4.0 mg/l BAP + 1.0 mg/l kinetin, T_{12}: MS + 0.5 mg/l BAP + 2.0 mg/l kinetin, T_{13}: MS + 1.0 mg/l BAP + 2.0 mg/l kinetin, T_{14}: MS + 2.0 mg/l BAP + 2.0 mg/l kinetin, T_{15}: MS + 4.0 mg/l BAP + 2.0 mg/l kinetin) were tested. The pH of the media was adjusted to 5.8 with pH meter (Elico-digital) using 1N HCL or 1N KOH/NaOH. Agar (8 g) was added to media and melted by placing the medium on hot plate. Twenty milliliters of medium poured into each of the culture tube (25mm x 150mm)/flask (100ml x 150ml), plugged with non-absorbent cotton wrapped in muslin cloth was autoclaved at 15 lb/inch^2 pressure for 20 minutes and solidification was done at room temperature.

Twenty centimeter long apical portion was taken from the current season's growth of the Perlette grape. Shoots were brought to the laboratory washed under running tap water for 15 minutes and 2 cm nodal segments were prepared from the middle of the shoot leaving the apical and basal nodes. The explants were thoroughly washed in running tap water for 30 minutes and then dipped in teepol (1%) for 10 minutes followed by a treatment with bavistin (0.05% - 0.1%) + diathane M-45 (0.25%) for 10 minutes. The explants were then rinsed in distilled water and treated with 70 per cent ethanol for 30-60 seconds. The explants were treated with 0.1 per cent mercuric chloride $HgCl_2$ under aseptic conditions and rinsed 4-5 times with sterilized distilled water. The explants were inoculated on to the culture media containing different concentrations of growth regulators under sterilized conditions and incubated in the culture room at 26±2^{0}C temperature, 55±5 per cent relative humidity and 16 hours continuous fluorescent light (2000 lux) followed by dark period of 8 hours. The data were recorded on the survival of explant and established cultures after four weeks of inoculation. After four weeks, the sprouted segments were taken out aseptically with the help of sterilized forceps from culture vessels. The optimum concentration and combination of the plant growth regulators for shoot proliferation was standardized by testing nine shoot proliferation treatments (M_1: MS + 0.5 mg/l BAP, M_2: MS + 1.0 mg/l BAP, M_3: MS + 2.0 mg/l BAP, M_4: MS + 0.5 mg/l BAP + 0.5 mg/l kinetin, M_5: MS + 1.0 mg/l BAP + 0.5 mg/l kinetin, M_6: MS + 2.0 mg/l BAP + 0.5 mg/l kinetin, M_7: MS + 0.5 mg/l BAP + 1.0 mg/l kinetin, M_8: MS + 1.0 mg/l BAP + 1.0 mg/l kinetin, M_9: MS + 2.0 mg/l BAP + 1.0 mg/l kinetin). The observations on per cent shoot regeneration, number of shoots per culture and shoot length (cm) were recorded after three subcultures (10 days each). The MS medium supplemented with different growth hormones was tested with six combinations (R1: MS alone, R_2: MS + 0.5 mg/l NAA), R_3: MS + 1.0 mg/l NAA, R_4: MS + 2.0 mg/l NAA, R_5: MS + 4.0 mg/l NAA and R_6: MS + 8.0 mg/l NAA) for rooting in plantlets. During one month of inoculation, observations were recorded for number of days taken for root initiation, number of roots per plantlet and the length of longest root was observed. In order to standardize the hardening protocol, the plants were transplanted four weeks after root initiation, into pots containing six different combinations of sand, soil, FYM and vermiculite (H_1: Sand alone, H_2: Sand (1 part) + FYM (1 part), H_3: Soil (1 part) + FYM (1 part), H_4: Sand (1 part) + soil (1 part) + FYM (1 part) + vermiculite (1 part), H_5: Sand (1 part) + soil (1 part) + vermiculite (1 part) and H_6: Sand (1 part) + soil (1 part) + FYM (1 part). Then they were subjected to hardening by covering the plants with polythene bags and glass beaker and maintaining humidity by providing mist spray of water over the plant by removing the cover while keeping in culture room continuously for 15 days in all treatments. For initial 3 days no air was allowed to enter and then little air space was provided in the cover. In all treatment, the plantlets were kept in continuous light (approx. 800 lux) in culture room at a temperature of 26 ± 2^{0}C. The cover was gradually removed after 6 days, initially for 3 hours followed by 6 hours and 12 hours in next 6 days. Subsequently, the period of keeping the plantlets at room temperature was gradually increased and / or increasing holes in the polythene bags with time, after 15 days they were brought outside air-conditioned room. The data were analyzed according to Completely Randomized Design (CRD) as described by Singh *et al.* (1998)

RESEARCH FINDINGS AND ANALYSIS

The findings of the study have been discussed in detail as under:

Explant establishment and shoot proliferation:

The data presented in Table 1 reveal significant variation for per cent explants establishment of Perlette grape in MS basal media fortified with different combinations of PBRs. The highest explant establishment (86.66%) and least days (12.00) for explants establishment were observed in MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l kinetin *i.e.* in T_9

Table 1: Effect of media on per cent culture establishment and days taken for culture establishment in explants of grape cv. Perlette

Treatments	Per cent culture establishment	Days taken for culture establishment
T_1	0.00 (0.00)	-
T_2	20.00 (26.55)	20.66
T_3	66.66 (54.96)	18.33
T_4	60.00 (50.74)	19.66
T_5	13.33 (17.70)	23.33
T_6	13.33 (17.70)	27.00
T_7	13.33 (17.70)	28.00
T_8	13.33 (17.70)	23.33
T_9	86.66 (72.26)	12.00
T_{10}	46.66 (43.06)	19.33
T_{11}	20.00 (26.55)	24.33
T_{12}	20.00 (26.55)	27.66
T_{13}	80.00 (63.40)	16.66
T_{14}	33.33 (34.99)	24.66
T_{15}	13.33 (17.70)	31.00
Mean	33.33 (32.50)	22.59
C.D.	9.95	0.77

* Figures in parenthesis are arc sine transformed values

media followed by T_{13} (80.00 per cent explant establishment in 16.66 days). The per cent explants establishment in T_9 media was statistically at par with T_{13} but days taken for explants establishment were statistically different for the two media. The explants did not establish in T_1 media (MS alone). The reason for no shoot regeneration on this media might be the lack of optimum cytokinin concentration required for adventitious shoot formation. The explants took the longest duration (31.00 days) for culture establishment while grown in T_{15} media (MS + 4.0 mg/l BAP + 2.0 mg/l kinetin). It seems that endogenous growth regulator level of the cultivar is the deciding factor for the optimum cytokinin concentration required to initiate shoots from explants. Pool and Powell (1975) found that cutting without roots must be provided with cytokinins to sustain the development of shoots. Lee and Wetzstein (1990) support these findings with their reports that adventitious shoot regeneration in muscadine grape cv. Summit was achieved when 10^{-5} M BAP was added to the medium. Addition of auxins along with BA/BAP improves the adventitious shoot formation in leaves of several grape cultivars while in others it was achieved on media supplemented with BAP only. The frequency was higher with 2 mg/1 BAP than with 1 or 4 mg/1 BAP with or without auxins. Kumar *et al.* (2008) reported 95.55 per cent shoot regeneration in nodal segment of grape in MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l kinetin. The results are in accordance with the findings of Gomes *et al.* (2004).

The data pertaining to shoot proliferation presented in Table 2 reveal significant variation for the number of days taken for shoot multiplication in grape cv. Perlette, however, on an average it took 30.55 days for explant multiplication. The lowest number of days (27.00) were taken for shoot proliferation in M_8 (MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l kinetin) which was statistically at par with 27.33 days taken in M_5 (MS + 1.0 mg/l BAP + 0.5 mg/l kinetin), however, maximum number of days (34.00) were taken while grown in M_1 (MS + 0.5 mg/l BAP). The highest number of shoots per explants (3.33) were obtained with M_5 media (MS + 1.0 mg/l BAP + 0.5 mg/l kinetin) followed by M_8 and M_2 without showing any significant difference. The minimum number of shoots per explants (0.33) were obtained in M_7 (MS medium supplemented with 0.5 mg/l BAP and 1.0 mg/l kinetin). Longest shoots (3.13 cm) were obtained in M_6 (MS + 2.0 mg/l BAP + 0.5 mg/l kinetin) followed by 2.93 cm in M_8 (MS + 1.0 mg/l BAP + 0.5 mg/l kinetin), while it was smallest (1.56 cm) with M_4 (MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l kinetin). Cytokinins are required for the proper development of shoots. Skene and Kerridge (1967) demonstrated a relationship between the cytokinin content of grape xylem sap and the

Table 2: Effect of media on shoot multiplication in grape cv. Perlette

Treatments	Number of days taken for multiplication	Number of shoots/ explants	Average shoot length (cm)
M_1	34.00	0.67	1.80
M_2	28.33	3.00	2.67
M_3	31.33	1.00	2.80
M_4	32.00	0.67	1.56
M_5	27.33	3.33	2.86
M_6	31.67	1.00	3.13
M_7	33.00	0.33	1.93
M_8	27.00	3.00	2.93
M_9	30.33	1.00	3.10
Mean	30.55	1.55	2.53
C.D.	1.37	0.88	0.20

vigour of shoot growth. In the present investigation, BAP was superior to kinetin for shoot proliferation and these results are in agreement to those of Baruah *et al.* (1996). Hu and Wang (1983) found that BA/BAP to be the most effective cytokinin for stimulating axillary shoot proliferation followed by kinetin and 2-iso-pentenyl adenine (2-iP).

***In vitro* rooting and plant establishment:**

A perusal of data (Table 3) shows significant variation for per cent *in vitro* rooting of Perlette grape. An average of 28.88 per cent rooting was obtained under different treatments being highest (73.33%) with R_3 (MS basal medium supplemented with 1.0 mg/l NAA) followed by 40.00 per cent rooting in R_4 (MS + 2.0 mg/l NAA). No rooting was observed in R_1 media (MS basal media alone). On an average, the plantlets took 39.66 days for root initiation. The plants grown in R_3 media (MS + 1.0 mg/l NAA) took the minimum number of days (26.33) for root initiation followed by 36.66 days in R_4 (MS+2.0 mg/l NAA). The highest number of days (49.00) were taken in R_6 (MS + 8.0 mg/l NAA). On an average, 1.53 roots per plantlet were formed under different treatments. The highest number of roots (2.66) were obtained in R_3 media (MS + 1.0 mg/l NAA) followed by 2.00 roots in MS + 2.0 mg/l NAA (R4). The minimum number of roots was one in R_2, R_5 and R_6. The maximum root length (4.43 cm) was obtained under R_3 (MS + 1.0 mg/l NAA) followed by 2.53cm in R_4 (MS + 2.0 mg/l NAA). The minimum root length was obtained in MS + 8.0mg/l NAA (R6). Hunter *et al.* (1984) found that IBA was the most desirable additive for root formation in strawberry, the optimum concentration being in the range of 0.75-1.0 mg/l, whereas Beura (2003) reported half strength MS medium supplemented with IBA 0.5-1. mg/l for *in vitro* rooting of horticultural crops. Likewise, different workers successfully obtained *in vitro* rooting in various fruit plants with different rooting hormones (Kaur, 2006 and Kabir *et al.*, 2007).

The data presented in Table 4 shows significant variation for per cent survival of *in vitro* raised plants of Perlette grape in different potting mixtures. The average survival was 34.44 per cent, showing its highest value (73.33%) was obtained in H_4 medium (sand (1part) + soil (1part) + FYM (1part) + vermiculite (1part)) followed by 53.33 per cent survival in H5 media (sand (1part) + soil (1part) + FYM (1part). No plant could survive in H1 media (sand only). The average increase in plant height of grape plantlets in potting media was registered as 1.25 cm and the highest increase (2.70 cm) was found in H_4 media (sand (1part) + soil (1part) + FYM (1part) + vermiculite (1part) followed by 2.13 cm in H_5 media (sand + soil + vermiculite, equal volumes). H_2 media (Sand + FYM, equal volumes) failed to increase any plant height during the course of present studies. The average increase in number of grape leaves was 1.46 cm registering its highest increase (3.33) was obtained in H_4 medium (sand + soil + FYM + vermiculite,

Table 3: Effect of media on *in vitro* rooting of grape cv. Perlette

Media code	Rooting (%)	Number of days taken for root initiation	Number of roots per plantlets	Length of root (cm)
R_1	0.00 (0.00)	-	-	-
R_2	26.66 (30.77)	40.33	1.00	0.93
R_3	73.33 (59.18)	26.33	2.66	4.43
R_4	40.00 (38.83)	36.66	2.00	2.53
R_5	20.00 (21.92)	46.00	1.00	0.56
R_6	13.33 (17.70)	49.00	1.00	0.43
Mean	28.88 (28.07)	39.66	1.53	1.78
C.D.	21.65	2.30	0.46	0.39

*Figures in parenthesis are arc sine transformed values

Table 4: Effect of potting mixture on *in vitro* raised plants of grape cv. Perlette

Treatments	Per cent survival	Initial plant height (cm)	Final plant height (cm)	Increased plant height (cm)	Initial no. of leaves	Final no. of leaves	Increased no. of leaves
H_1	0.00 (0.00)	-	-	-	-	-	-
H_2	20.00 (21.92)	3.06	3.06	0.00	3.00	3.33	0.33
H_3	26.66 (30.77)	3.00	3.56	0.56	3.00	4.00	1.00
H_4	73.33 (59.18)	3.03	5.73	2.70	3.00	6.33	3.33
H_5	53.33 (46.90)	3.10	5.23	2.13	3.00	5.67	2.67
H_6	33.33 (34.99)	3.06	3.93	0.86	3.00	3.00	0.00
Mean	34.44 (32.29)	3.05	4.30	1.25	3.00	4.46	1.46
C.D.	17.86			0.19			0.82

* Figures in parenthesis are arc sine transformed values

equal volumes), however, no increase in number of leaves could be observed in H_6 medium. For hardening of *in vitro* raised fruit plants different workers have suggested different mediums such as soil-vermiculite mixture (Goyal and Arya, 1981), soil (Kurten *et al.*, 1990) and sterilized soil (Bhansali *et al.*, 1988). Among various potting mixtures tried, the mixture containing sand (1 part) + soil (1 part) + FYM (1 part) + vermiculite (1 part) was found to be the most suitable. Physical, chemical and biological properties of the potting mixtures display a vital role in the establishment of *in vitro* produced plantlets. Better performance of FYM might be attributed to its ability to improve biological properties of the soil. On the other hand, sand may be responsible for providing sufficient aeration and high water holding capacity provided by vermiculite. Hence, mixing sand, soil, FYM and vermiculite in equal volumes might have helped in giving better grip for the roots, ample aeration and sufficient organic matter. Similar results have also been obtained in different crops (Kaur *et al.*, 2005 and Singh *et al.*, 2002).

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