OPTIMIZING THE CONCENTRATIONS OF PLANT GROWTH REGULATORS FOR *IN VITRO* SHOOT CULTURES, CALLUS INDUCTION AND SHOOT REGENERATION FROM CALLUSES OF GRAPES

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Abstract

Aim: To optimize the concentrations of growth regulators in the media for the proficient micropropagation of grapevine (*Vitis vinifera* L.) cv. King's Ruby.

Methods and results: Apical meristems of the cultivar were used to establish in vitro shoot cultures. Nodal explants from these shoots were inoculated in half strength Murashige and Skoog (MS) shoot proliferation media supplemented with benzyl aminopurine (BAP), kinetin, glycine and gibberellic acid (GA3). A higher number of shoots (5.33) with greater length (2.75 cm) was produced in the medium supplemented with 1.0 mg L-1 BAP and 0.1 mg L^{-1} GA3. Callus induction from leaf explants taken from in vitro grown shoots was greater (73.00%) on the medium containing 2.0 mg $^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3 mg L-1 BAP and 0.2 mg L^{-1} α-naphthaleneacetic acid (NAA). The maximum shoot regeneration frequency (53.33%) was achieved on the medium supplemented with 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, and the rooting of regenerated shoots was successful on growth regulator-free half strength MS medium.

Conclusion: Optimizing the BAP and GA3 and omitting the glycine and kinetin in the culture medium increased the number and length of shoots. We recommended to use higher 2,4-D and lower BAP concentrations in the medium for callus induction of the leaf explants taken from in vitro grown shoots, and relatively high levels of BAP and NAA (1.5 and 0.5 mg L^{-1}, respectively) for maximum shoot regeneration. Finally, we suggest the half strength growth regulators-free MS medium for the rooting of regenerated shoots.

Significance and impact of the study: Optimizing the concentration of growth regulators is crucial to establish in vitro shoot cultures, induce calluses, regenerate shoot and, hence, to propagate disease-free true to type grape cultivars in a short time.

Key words: micropropagation, tissue culture, *Vitis vinifera*, shoot proliferation, plantlet regeneration

Abbreviations: BAP = benzyl aminopurine, 2,4-D = 2,4-dichlorophenoxyacetic acid, GA_3 = gibberellic acid, MS = Murashige and Skoog, NAA = α-naphthaleneacetic acid

Résumé

Objectif: Optimiser la concentration de régulateurs de croissance dans les milieux de culture afin d'améliorer la « micropropagation » de la vigne (*Vitis vinifera* L.) cv. King's Ruby.

Méthodes et résultats: Des méristèmes apicaux du cépage ont été utilisés pour établir des cultures *in vitro*. Des explants nodaux provenant de ces pousses ont été ensemencés dans un milieu de prolifération, c'est-à-dire un milieu Murashige et Skoog (MS) dilué de moitié, avec ajout de benzyle aminopurine (BAP), de kinétine, de glycine et d'acide gibbérellique (GA_3). Le plus grand nombre (5.33) et les plus longues pousses (2.75 cm) ont été obtenus dans le milieu additionné de 1.0 mg L^{-1} BAP et 0.1 mg L^{-1} GA_3. La formation de cals a été induite à partir d'explants de feuille prélevés de pousses cultivées *in vitro*. La production de cals a été la plus forte (73.00 %) sur le milieu contenant 2.0 mg L^{-1} d'acide 2,4-dichlorophénoxyacétique (2,4-D), 0.3 mg L^{-1} de BAP et 0.2 mg L^{-1} d'acide α−naphtalèneacétique (NAA). La fréquence maximale de régénération de pousses (53.33 %) a été obtenue sur le milieu additionné de 1.5 mg L^{-1} BAP et 0.5 mg L^{-1} NAA, et les pousses régénérées ont formé avec succès des racines dans le milieu MS dilué de moitié, sans ajout de régulateurs de croissance.

Conclusion: L'optimisation de la concentration en BAP et GA_3 et l'omission de la glycine et de la kinétine dans le milieu de culture ont permis d'augmenter le nombre et la longueur des pousses.

Signification et impact de l'étude: Optimiser la concentration des régulateurs de croissance est essentielle pour la « micropropagation » efficace d'un cépage. Connaître l'équilibre spécifique entre les régulateurs de croissance est nécessaire afin d'établir des cultures de pousses *in vitro*, l'induction des cals et la régénération des pousses, et ainsi multiplier rapidement des cépages exempts de maladie et conformes au type variétal.

Mots clés: micropropagation, culture de tissus, *Vitis vinifera*, prolifération des pousses, régénération des plantules

Abréviations: BAP = benzyle aminopurine, 2,4-D = acide 2,4-dichlorophénoxyacétique, GA_3 = acide gibbérellique, MS = Murashige et Skoog, NAA = acide α−naphtalèneacétique

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INTRODUCTION

The grape (*Vitis vinifera* L.), a globally cultivated commercial fruit crop, is native to warm and temperate zones. The *Vitis* genus is widely distributed between 25° and 50° N latitude in eastern Asia, Europe, Middle East and North America (Sajid *et al*., 2006). The grape is consumed as a fresh and dried fruit or used for making drugs, jam, vinegar, juice, jelly and wine. The global economic impact of the grape, grape juice and wine industry represents billions of dollar. Being a good source of basic food components, minerals and vitamins, grapes prevent various human diseases through their antioxidant activity and show antitumor activities by blocking carcinogen-induced DNA adduct formation (Jung *et al*., 2006).

However, the economic and health benefits of grape could be threatened by many serious diseases including fungal (powdery mildew and gray rot), viral (fan leaf roll fleck, stem pitting and corky bark) and bacterial diseases (pierces and necrosis) that are accountable for the low yield and shortened life span of grapevine (Jaskani *et al*., 2008). These diseases mainly originate from the infected propagating material obtained from conventional grapevine propagation methods. Further, these conventional methods are time consuming. However, the risk of infection can be eliminated through unconventional propagation techniques like micropropagation or tissue culture, which ensures the mass production of virus- and disease-free « elite » planting material.

Micropropagation is the art and science of *in vitro* plant multiplication. Generally, the plants that do not produce seeds or do not respond well to traditional vegetative propagation are multiplied through this method. A small piece of tissue is removed from a stock plant and grown in a nutrient medium under controlled aseptic physical conditions to produce numerous novel plants or plantlets. A large number of uniform true to type disease-free plants are produced in a relatively short time and space because this process is independent of the season and weather conditions. The prevalent micropropagation techniques include shoot proliferation, organogenesis and somatic embryogenesis. Shoot proliferation is achieved through meristem or meristem tip cultures and axillary bud cultures. In grape, shoot cultures have been established from nodal segments containing a single axillary bud (Mhatre *et al*., 2000). Callus has been successfully initiated from explants of different origins and, moreover, grape cultivars have been propagated through somatic embryogenesis and shoot regeneration (Das *et al*., 2005 ; Cadavid-Labrada *et al*., 2008 ; Malabadi *et al*., 2010 ; Diab *et al*., 2011).

Shoot regeneration from fragmented shoot apices has been profitably applied to several grape species and hybrids (Barlass and Skene, 1978 ; Salami *et al*., 2005 ; Sajid *et al*., 2006). However, medium composition, especially growth regulators and concentrations used, directly affects the growth and *in vitro* propagation time of explants. In plants, growth regulators have pleiotropic effects and small changes in the concentrations result in gene activation shifts that may hinder or initiate essential metabolic processes within the cell. Hence, an optimal concentration of plant growth regulators is necessary for normal cell functioning. Similarly, optimizing the concentrations of different plant growth regulators and their combinations in the medium is a prerequisite of *in vitro* propagation (Thomas, 2000) as this hormonal balance determines certain developmental pathways of a plant cell. Therefore, the aim of the present investigation was to optimize the levels of various plant growth regulators for establishing *in vitro* shoot cultures, callus induction and plantlet regeneration from calluses and to develop an efficient protocol for the micropropagation of grape (*Vitis vinifera* L.) cv. King's Ruby.

MATERIALS AND METHODS

The research was conducted in the Laboratory of Tissue Culture, Department of Horticulture, PMAS-Arid Agriculture University, Rawalpindi. Tissue culture reagents and general chemicals (analytical grade) were purchased from either Sigma-Aldrich (Saint Louis, USA) or Merck (Darmstadt, Germany).

1. Plant material and methodology for explant culture

Shoot tips were collected from *Vitis vinifera* L. cv. King's Ruby plants grown in the field. The tips were first rinsed with Tween 20 detergent for 30 minutes, then soaked in 70 % ethanol for 1 minute and surface disinfected with 0.1 % mercuric chloride ($HgCl_2$) solution for 7 minutes. Afterwards, these were washed 3–4 times with autoclaved distilled water, 5 minutes each time, in a laminar flow hood. Apical meristems, 0.2 mm in size, were excised from the tips with the help of a micro scalpel under a stereo dissecting microscope in a laminar flow hood. The hood and instruments were properly illuminated and sterilized for this purpose.

2. Culture establishment

After excision, the apical meristems were immediately cultured in a test tube containing 25 mL of MS (Murashige and Skoog, 1962) medium (MS macro- and micro-elements and vitamins) without supplementing any growth regulator. The pH was adjusted to 5.8 ± 0.1 before sterilization at 121 °C and 102.97 kPa for 20 minutes. The cultures were incubated at 25 ± 1 °C with a 16-h photoperiod under 3,000 lux light intensity provided by cool-white fluorescent tubes (Philips, Netherlands). The cultures were periodically examined and visually observed for necrosis, bacterial and fungal contamination, and explant survival rate.

3. Shoot multiplication

After 3–4 weeks of establishment of *in vitro* shoot cultures, the healthy shoots were harvested and divided into 1.0–1.5-cm nodal segments, each containing an axillary bud. The segments were transferred to culture jars containing half strength MS medium supplemented with gibberellic acid (GA_3; 0.1, 0.3 or 0.5 mg L^{-1}) and either benzyl aminopurine (BAP; 1.0, 1.5 or 2.0 mg L^{-1}), kinetin (0.5, 1.0 or 1.5 mg L^{-1}) or glycine (1.0, 1.5 or 2.0 mg L^{-1}); control medium did not contain any growth regulator (Table 1). All the media were fortified with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar. The cultures were kept at 25 ± 1 °C with a 16-h photoperiod under 2,000 lux light intensity to induce the formation of multiple shoots (Wei *et al*., 1994) and to promote organ differentiation and plant growth (Villegas and Bravato, 1991). The data on the number and length of shoots were recorded after four weeks of culture.

4. Callus induction

Leaves were harvested from *in vitro* grown shoot cultures and leaf discs (each with an area of 0.5 cm^2) were prepared. Leaf discs were placed on MS medium supplemented with 30 g L^{-1} sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D), BAP and α-naphthaleneacetic acid (NAA) at concentrations indicated in Table 2, and solidified with 7 g L^{-1} agar. All the cultures were maintained in the dark at 25 ± 2 °C. The frequency of callus induction was estimated as follows:

$$\text{Callus induction frequency (\%)} = \frac{\text{Number of calluses induced}}{\text{Number of leaf discs inoculated}} \times 100$$

5. *In vitro* shoot regeneration

After 2–3 subcultures, the calluses were aseptically shifted to the shoot regeneration medium in test tubes. Regeneration medium was composed of MS medium supplemented with 30 g L^{-1} sucrose and BAP and NAA at concentrations indicated in Table 3, and solidified with 7 g L^{-1} agar. The cultures were incubated at 25 ± 1 °C with a 16-h photoperiod under 3,000 lux light intensity. The frequency of shoot regeneration was calculated as follows:

$$\text{Shoot regeneration frequency (\%)} = \frac{\text{Number of calluses regenerated into shoots}}{\text{Total number of calluses inoculated}} \times 100$$

6. Statistical analysis

The experiments were arranged in Completely Randomized Design (CRD) with three replications. The data obtained were statistically analyzed by using Statistix 8.1 analytic software (Tallahassee Florida, USA). The means were compared by least significance difference (LSD) test at $p = 0.05$.

RESULTS AND DISCUSSION

1. *In vitro* culture establishment

Fungal contamination of grapevine explants is a serious problem and explants taken from the field are often contaminated with bacteria and fungi. Hence, surface disinfection of the explants is a prerequisite for sterilized culture establishment. Therefore, in the present study, shoot tip explants − taken from field grown plants − were disinfected with 0.1 % $HgCl_2$ for 7 minutes. Afterwards, meristems were excised and cultured on nutrient medium under aseptic conditions. Apical meristems presumably contain less endophytic contamination than other plant tissues (Gray and Benton, 1991) and vigorously grow when cultured *in vitro*.

2. Growth of *in vitro* shoot cultures

The genotype and size of explants and the nature and concentrations of plant growth regulators are the limiting factors in the micropropagation of grapevines and other woody species (Yerbolova *et al*., 2013). In the present study, shoot number was significantly affected by the different combinations of growth regulators. For shoot proliferation, a nodal segment containing an axillary bud was taken from the *in vitro* grown shoots and cultured on half strength MS medium supplemented with the different combinations of plant growth regulators: glycine, kinetin, BAP and GA_3. The combination of BAP and

GA_3 (1.0 and 0.1 mg L^{-1}, respectively) resulted in the highest (5.33) number of shoots per cultured explant, followed by the combinations BAP (1.5 mg L^{-1}) + GA_3 (0.3 mg L^{-1}) and BAP (2.0 mg L^{-1}) + GA_3 (0.5 mg L^{-1}), which produced 4.33 and 4.05 shoots per culture, respectively (Table 1). Ibanez *et al*. (2005) also reported similar findings, showing that the medium containing 1 or 2 mg L^{-1} BAP plays an important role in the development of axillary buds and shoots in explants. Similar results were reported by Mukherjee *et al*. (2010). Besides, the increase in the concentration of BAP in the basal MS medium increased the shoot multiplication rate of *in vitro* cultures of grape cultivars/accessions (Heloir *et al*., 1997; Sajid *et al*., 2006; Tehrim *et al*., 2013). BAP also yielded the maximum shoot number in grapevine cv. Perlette (Jaskani *et al*., 2008) and in orchid (Asghar *et al*., 2011) when propagated *in vitro*. Abido *et al*. (2013) tested 1.0, 2.0, 3.0 and 4.0 mg L^{-1} BAP, 0.1, 0.2 and 0.3 mg L^{-1} NAA and their combinations for shoot multiplication of *Vitis vinifera* L. cv. Muscat of Alexandria. The maximum number of proliferated shoots was obtained on MS medium containing 3.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA. Further, Butiuc-Keul *et al*. (2009) and Craciunas *et al*. (2009) found that supplementing the culture medium with cytokinins improved the shoot multiplication rate of grapevine, possibly because cytokinins stimulate cell division and promote axillary shoot growth in tissue cultures (Gray *et al*., 2005).

In the present study, the combinations of glycine and GA_3 produced 2.00 to 3.33 shoots per culture, which was less than those produced by the media supplemented with BAP and GA_3; moreover, the shoots were stunted and distorted. The media supplemented with combinations of kinetin and GA_3 resulted in depressed growth with 1.33 to 1.58 shoots per culture, whereas the medium without any growth regulator (control) produced only 1.16 shoots per cultured explant. On the whole, the number of shoots increased due to the addition of growth regulators, which had a positive effect on shoot proliferation and multiplication. The results also depict a strong interaction between growth regulators and mineral elements. Axillary shoots grew (5.33 shoots per culture) on half strength MS medium supplemented with 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} GA_3 (Fig. 1a). This might be due to the interaction between growth regulators and macro- and micro-nutrients resulting in better growth and multiplication of the cells. *In vitro* culture of *Vitis vinifera* was established on media without growth regulators by Galzy (1961). Afterwards, Harris and Stevenson (1982) found that supplementation of a cytokinin to the medium improved shoot multiplication (Butiuc-Keul *et al*., 2009). In the present investigation, BAP was superior to kinetin and glycine for shoot proliferation. BAP (i) is considered the most effective cytokinin for the stimulation of axillary shoot proliferation, followed by kinetin (Hu and Wang, 1983), (ii) produces high quality shoots, compared to kinetin which is ineffective and comparable to control (Gray and Benton, 1991) and (iii) induces shoot development in *Vitis* spp. (Diab *et al*., 2011). In fact, growth regulators, used either alone or in different combinations, define the success of *in vitro* shoot proliferation of grapevine (Aazami, 2010) and efficient micropropagation depends on this rapid and uniform shoot proliferation.

As for shoot growth, the culture medium supplemented with 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} GA_3 achieved the maximum shoot length (2.75 cm), followed by the media with 1.5 mg L^{-1} BAP + 0.3 mg L^{-1} GA_3 and with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} GA_3 (1.44 and 1.06 cm, respectively) (Table 1). The shoot

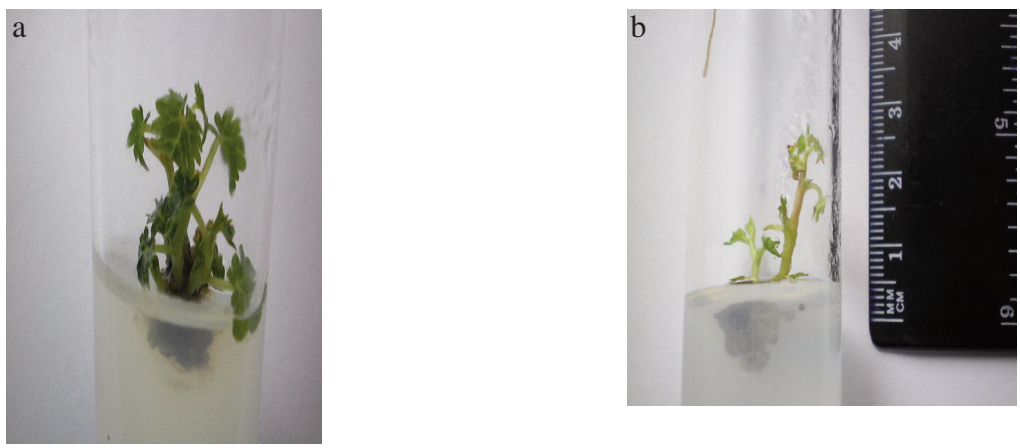

Figure 1. (a) Shoot number and (b) shoots length obtained on the culture medium supplemented with 1.0 mg L^{-1}BAP and 0.1 mg L^{-1} GA3.

elongation phase is sensitive to higher concentrations of growth regulators (Kadota and Niimi, 2003). In the present study, the combination 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} GA_3 significantly increased shoot length. However, a high concentration of BAP restricted shoot elongation, which might be due to its toxic level. Sajid *et al*. (2006) and Tehrim *et al*. (2013) also observed a decrease in the shoot length of grape accessions with increasing concentrations of BAP. A high concentration of cytokinins results in ethylene production that limits the regeneration of shoots and inhibits the elongation of internodes. Further, in the present study, glycine and kinetin inhibited shoot elongation and multiplication. All the combinations of kinetin and glycine with GA_3 resulted in small and distorted shoots. Explant growth depends on the nutrients and growth regulators added to the culture medium. The combination of various growth regulators and their concentrations significantly influences shoot length due to their effect on cell division and cell expansion (Gordon and Letham, 1975). Therefore, growth regulators are noticeably important for *in vitro* shoot proliferation, but some internal factors and nutrient conditions can modify their activities (Park *et al*., 2001). Shoot regeneration also depends upon endogenous levels of growth regulators in the explant and the position of node, as different types of buds showed different regeneration efficiencies in grape (Nontaswatsri *et al*., 2002). The present study indicates that BAP in combination with GA_3 effectively elongated the shoots (Fig. 1b).

3. Callus induction

Leaf discs taken from *in vitro* shoot cultures were used for callus induction. Leaf discs were placed on MS medium supplemented with the different concentrations of plant growth regulators (2,4-D, BAP and NAA). NAA was used at a constant rate of 0.2 mg L^{-1}. The higher concentration of 2,4-D (2.0 mg L^{-1}) combined with the lower concentration of BAP (0.3 mg L^{-1}) induced the highest number of calluses (73.00 %), followed by the mid-level of growth regulators (1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP), which induced 51.00 % calluses. Moreover, the lower level of 2,4-D (1.0 mg L^{-1}) combined with the higher level of BAP (0.7 mg L^{-1}) resulted in lower callus induction frequency (31.00 %). The control treatment (with no growth regulator) induced the lowest (6.66 %) frequency of callus induction (Table 2), probably due to the lack of optimum auxin concentration required for the induction. Thus, in the present study, the higher concentration of 2,4-D induced more calluses from the leaf discs compared to the lower concentrations. The higher concentration of 2,4-D combined with the lower concentrations of BAP and NAA favored callus formation (Fig. 2). Decreasing the concentration of 2,4-D and increasing that of BAP reduced callus formation rate. It is further revealed that 2,4-D, even at the lowest concentration (1.0 mg L^{-1}), induced more calluses than the control but the process took a minimum of six weeks (data not shown). Hence, NAA and BAP did not stimulate explants to initiate growth and form callus as compared to 2,4-D. However, the small amount of BAP (0.2 mg L^{-1}) had a positive effect on callus induction. BAP promotes RNA and protein

Table 1. Effect of different concentrations of various PGRs on shoot proliferation

Growth regulator combinations	Number of shoots produced	Shoots length (cm)
Control (without any growth regulator)	1.16 ^{e}	0.29 ^{f}
BAP + GA_3		
1.0 mg L^{-1} BAP + 0.1 mg L^{-1} GA_3	5.33 ^{a}	2.75 ^{a}
1.5 mg L^{-1} BAP + 0.3 mg L^{-1} GA_3	4.33 ^{b}	1.44 ^{b}
2.0 mg L^{-1} BAP + 0.5 mg L^{-1} GA_3	4.05 ^{bc}	1.06 ^{bc}
Kinetin + GA_3		
0.5 mg L^{-1} Kinetin + 0.1 mg L^{-1} GA_3	1.58 ^{de}	0.72 ^{cd}
1.0 mg L^{-1} Kinetin + 0.3 mg L^{-1} GA_3	1.33 ^{e}	0.75 ^{cd}
1.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} GA_3	1.58 ^{de}	1.00 ^{c}
Glycine + GA_3		
1.0 mg L^{-1} Glycine + 0.1 mg L^{-1} GA_3	3.33 ^{c}	0.86 ^{cd}
1.5 mg L^{-1} Glycine + 0.3 mg L^{-1} GA_3	2.41 ^{d}	0.93^{cd}
2.0 mg L^{-1} Glycine + 0.5 mg L^{-1} GA_3	2.00 ^{de}	0.61^{de}
$LSD_{(0.05)}$	0 89	0 39

Data represent mean of 3 repeats.

Table 2. Effect of different concentrations of various plant growth regulators on callus induction

Growth regulator combinations	Number of explants cultured	Number of calluses induced	Frequency (%) of callus induction
0.0 mg L^{-1} 2,4-D + 0.0 mg L^{-1} BAP + 0.0 mg L^{-1} NAA	45	3	6.66 ^{d}
1.0 mg L^{-1} 2,4-D + 0.7 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	45	14	31.00 ^{c}
1.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	45	23	51.00 ^{b}
2.0 mg L^{-1} 2,4-D + 0.3 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	45	33	73.00 ^{a}
LSD $_{(0.05)}$			12.00

Data represent mean of 3 repeats.

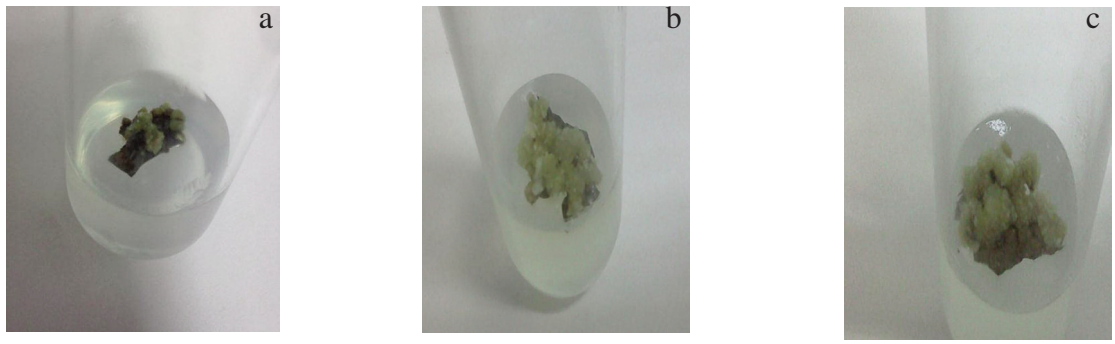

Figure 2. Callus induction on the culture medium supplemented with 2,4-D, BAP and NAA @ 2.0 + 0.3 + 0.2 mg L^{-1} (a) one week of initiation of callus, (b) after three weeks and (c) explant fully covered with callus after four weeks of culture.

synthesis which activates enzyme activity for cell division and cell wall loosening (Kulaeva, 1980).

The present results are in line with the findings of Hasbullah *et al*. (2011), who stated that 1.0 to 2.0 mg L^{-1} 2,4-D with a small concentration of NAA and BAP produced callus in *Gerbera jamesonii* when subcultured at 2-week intervals. Further, these authors also observed the highest callus induction rate at the higher level of 2,4-D; however, minutely decreasing the concentration of 2,4-D kept reducing the callus induction rate. Thus, the combinations of three growth regulators (2,4-D, NAA and BAP) had a positive effect on callus induction and, moreover, this study suggests that 2,4-D with the lower concentrations of NAA and BAP constitutes the most favorable combination of growth regulators for the production of callus in grapevine cv. King's Ruby. These findings are supported by Can *et al*. (2008), who found that higher concentrations of auxins and cytokinins inhibited meristematic cell division and callus induction and decreased chemical reactions, resulting in the stunted growth and finally the death of explants.

4. Shoot regeneration

Regeneration of grapevine is possible through both organogenesis and embryogenesis. Explants including shoot tips, floral buds, leaves and tendrils can be regenerated into somatic embryos directly or indirectly through a callus phase (Salunkhe *et al*., 1997; Aazami, 2010). Plant organs like shoot apices, anthers, zygotic embryos, ovaries, tendrils and leaves are often *in vitro* cultured because these are readily available and can easily be manipulated (Salunkhe *et al*., 1997).

In the present study, regeneration was processed through calluses of leaf origin. A significantly different number of shoots was formed in response to the combinations of BAP and NAA (Table 3). The medium with the highest concentrations of BAP and NAA (1.5 and 0.5 mg L^{-1}, respectively) achieved the maximum shoot regeneration frequency (53.33 %). However, decreasing the concentrations to 1.0 mg L^{-1} BAP + 0.3 mg L^{-1} NAA decreased the regeneration frequency to 26.67 % and further decreasing the concentrations (0.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA) led to a further decrease in the regeneration frequency (16.67 %), finally reaching the lowest shoot regeneration frequency (6.67 %) in control medium (without any growth regulator).

Callus necrosis was observed with increasing concentrations of growth regulators in the regeneration medium. Green spots appeared on the calluses and shoots formed with the application of

Table 3. Effect of different concentrations of various PGRs on shoot regeneration

Growth regulator combinations	Number of explants cultured	Number of plantlets formed	Frequency (%) of regeneration
0.0 mg L^{-1} BAP + 0.0 mg L^{-1} NAA	30	2	6.67 ^{cd}
0.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	30	5	16.67 ^{bc}
1.0 mg L^{-1} BAP + 0.3 mg L^{-1} NAA	30	8	26.67 ^{b}
1.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	30	16	53.33 ^{a}
$LSD_{(0.05)}$			17.19

Data represent mean of 3 repeats.

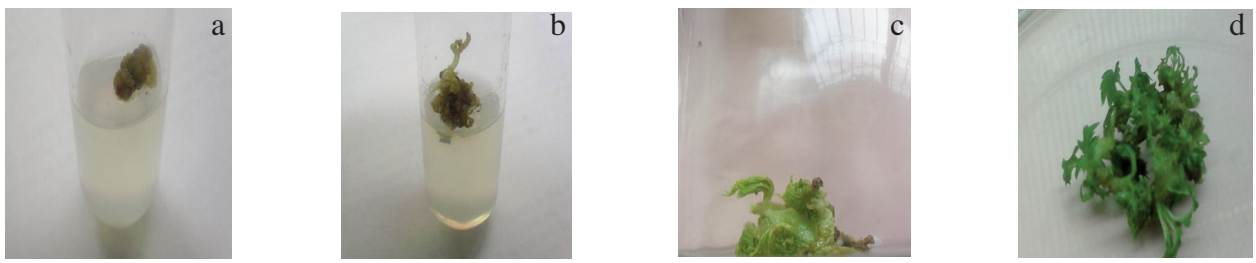

Figure 3. Shoot regeneration on the culture medium containing BAP and NAA @ 1.5 + 0.5 mg L^{-1} (a) after two weeks, (b) three weeks, (c) four weeks and (d) six weeks of culture.

BAP and NAA at the rate of 1.5 and 0.5 mg L^{-1}, respectively (Fig. 3). The lower concentrations of BAP and NAA (1.0 and 0.3 mg L^{-1}, respectively) lowered the regeneration rate. Thus, it was concluded that shoot regeneration of grapevine cv. King's Ruby from calluses of leaf origin is sensitive to auxins and cytokinins, and that a specific concentration of auxins and cytokinins is required for optimum shoot regeneration.

The regenerated shoots readily formed roots when transferred to half strength MS medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar without any growth regulator and kept at 25 ± 1 °C with a 16-h photoperiod under 2,000 lux light intensity.

CONCLUSION

The number and length of shoots were maximum in the culture medium optimized with the combination of 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} GA_3. However, the combinations of GA_3 with glycine or kinetin resulted in less shoots that were stunted and distorted as well. Similarly, the highest callus induction of leaf explants taken from *in vitro* grown shoots could be obtained on a medium containing a combination of the higher concentration of 2,4-D and the lower concentration of BAP (2.0 mg L^{-1} and 0.3 mg L^{-1}, respectively). Moreover, increasing the concentration of 2,4-D and decreasing that of BAP favored callus induction. The medium was also optimized for shoot regeneration and it was observed that the highest concentrations of BAP and NAA (1.5 and 0.5 mg L^{-1}, respectively) achieved the maximum shoot regeneration frequency. Furthermore, decreasing the concentration of both also reduced the number of regenerated shoots. Finally, it is recommended to use half strength MS medium with no growth regulator for the rooting of regenerated shoots.

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