EFFECTS OF COLD PRETREATMENT, CARBOHYDRATE SOURCE AND GELLING AGENTS ON SOMATIC EMBRYOGENESIS FROM ANTHERS OF *VITIS VINIFERA* L. CVS. 'REGINA' AND 'REICHENSTEINER'

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Abstract

The effects of cold pretreatment of inflorescences (1, 2, or 3 weeks at 4°C), different carbon sources (sucrose, glucose, maltose, galactose and mannitol) and two gelling agents (agar at 0.7% and Gelrite at 0.25%) were investigated for their effects on callus formation and somatic embryogenesis in anther tissues of *Vitis vinifera* L., cvs. Regina and Reichensteiner. In cv. Regina, cold pretreatment for 2 weeks promoted development of more embryogenic explants, improved embryogenic efficiency and increased the number of germinated somatic embryos compared with 1, or 3 weeks cold pretreatment. Sucrose produced the highest callus formation followed by sucrose + mannitol, glucose and maltose. Mannitol did not support callus formation. Somatic embryogenesis occurred on the sucrose- and glucose-supplemented media. In cv. Reichensteiner, carbon source also significantly affected callus formation and somatic embryogenesis, which again occurred on sucrose- and glucose-supplemented media, However, embryogenic efficiency and embryo germination were higher among embryos cultured on glucose medium than on sucrose medium. The type of gelling agent did not have a significant impact on callus formation in cv. Reichensteiner during 4 weeks of culture in the dark. Anthers cultured on agar, or Gelrite produced somatic embryos with a similar embryogenic efficiency. In all of these experiments, normal and abnormal somatic embryos were obtained.

Keywords: anther culture, carbohydrates, gelling agents, grape, *Vitis vinifera*

1. Introduction

In grape (*Vitis vinifera* L.), somatic embryogenesis was first reported by using nucellar tissue of unfertilised ovules. Other reports of successful embryogenesis used anthers (Rajasekaran and Mullins, 1979), ovaries (Gray and Mortensen, 1987), zygotic embryos (Stamp and Meredith, 1988b), immature leaves and stems (Krul and Worley, 1977) and mature leaves (Stamp and Meredith, 1988a). The vast majority of published work on induction of somatic embryogenesis in grape has focused on economically important wine cultivars such as Cabernet-Sauvignon (Mullins and Srinivasan, 1976; Gray and Mortensen, 1987), with little work on table grapes. Several factors have been found to affect somatic embryogenesis such as anthers cold pretreatment, carbon source and gelling agents. In grape, chilling flowers before anther dissection may result in an increased frequency of embryogenic anthers (Rajasekaran and Mullins, 1979). Reports on the use of cold pretreatment of anthers mostly recommend 3 days of chilling (Mauro et al., 1986; Gray and Mortensen, 1987; and Perl et al., 1995), or occasionally 4 days (Harst and Alleweldt, 1993). Some workers use no chilling pretreatment at all (Stamp and Meredith, 1988a). Sucrose is the most commonly used carbohydrate for plant tissue culture (Tisserat et al., 1979; Evans et al., 1981). It is also the main carbon source used for the initiation of embryogenic callus in many plant species, including grape (Stamp and Meredith, 1988b; Emershad and Ramming, 1994) and has been used at various concentrations ranging from 1% (Mozsar and Sule,

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1994), to 6% (Emershad and Ramming, 1994; Perl et al., 1995). Agar is widely used as a gelling agent in plant tissue culture work (Torres, 1989). Most of the studies on induction of somatic embryogenesis in grape have utilised agar as a solidifying agent, at concentrations which ranged from 0.65% (Emershad and Ramming, 1994), to 0.8% (Mozsar and Sule, 1994; Robacker, 1993). Gelrite was occasionally used at 0.25% (Perl et al., 1995), or 0.3% (Harst, 1995). Yellow anther colour (Stamp and Meredith, 1988a), or anther length (0.5 - 0.7 mm) (Mauro et al., 1986), were among the most important practical indicators of embryogenic competence.

The objectives of the present study were to test the effects of cold pretreatment, carbohydrate source and type of gelling agent on induction of somatic embryogenesis in table grapes cvs. Regina and Reichensteiner.

2. Materials and methods

2. 1. Culture conditions

In all experiments, media were supplemented with 2,4-D at 4.5 μM and BA at 1.0 μM. The cultures were incubated for 4 weeks in the dark at 24°C and observations were made weekly on survival rate and callus formation. Callused explants were subcultured onto B5 growth regulator-free medium and placed in the light and observed for appearance of somatic embryos. Somatic embryogenesis was evaluated by counting the number of embryogenic explants and calculating embryogenic efficiency (number of embryos per explant). Somatic embryos were germinated on B5 medium solidified with 0.7% agar. Germinated embryos (with cotyledons and true leaves) were counted and transplanted into Jiffy-7 planting blocks and placed in a propagation box where relative humidity was reduced gradually by venting. Acclimatised plantlets were transplanted into 10 cm pots containing potting compost and placed in the greenhouse under natural lighting.

2. 2. Cold pretreatment

Flower clusters of cv. Regina were collected from one vine and chilled at 4°C for 1, 2, or 3 weeks. They were surface sterilised by immersion in sodium hypochlorite at 1.0% (available Cl) with a few drops of "Tween 20" for 10 min with continuous agitation and rinsed in sterile distilled water three times, for 5 minutes each. Anthers were dissected under a binocular microscope and cultured on B5 medium (Gamborg, 1968).

2. 3. Carbohydrate source

Flowers of cvs. Regina and Reichensteiner were surface sterilised as stated above and anthers were placed on B5 medium. For cv. Regina, sucrose, maltose and mannitol at 2%, glucose at 1.5% and sucrose + mannitol at 2% each were used. For cv. Reichensteiner, sucrose, galactose, glucose and maltose were used at 2%. After 4 weeks explants were subcultured onto B5 growth regulator-free medium containing the same carbon source.

2. 4. Type of gelling agent

To investigate the effect of gelling agents on somatic embryogenesis in anthers of cv. Reichensteiner, agar (Difco-Bacto) at 0.7% and Phytagel "Gelrite" (Sigma) at 0.25% were used. Flower clusters were surface-sterilised as before and anthers were cultured on B5 medium solidified with one of the gelling agents.

2. 5. Design of experiments

All experiments consisted of 20 replicates per treatment (one dish per replicate) with five explants each, in a completely randomised design. Data were analysed using Analysis of Variance (ANOVA) and Duncan's multiple range test using the SAS program (GLM procedure, SAS Institute Inc., 1985) release 6.07.

3. Results

3. 1. Effects of cold pretreatment

Anthers of cv. Regina which were kept for 1, 2, or 3 weeks at 4°C and following callus initiation, produced somatic embryos after 3-4 weeks when subcultured into growth regulator-free medium. Somatic embryos passed through globular, heart, torpedo and cotyledonary stages. The percentage of anthers producing somatic embryos ranged from 0.7 to 2.1% (Table 1). Cold pretreatment for 1 week stimulated the most callus formation, however, the 2 week pretreatment produced the highest number of embryogenic explants and germinated embryos.

Table 1. Effects of cold pretreatment of anthers on callus formation and somatic embryogenesis in cv. Regina.

Pretreatment (wks at 4°C)	callus formation (%)	embryogenic explants (%)	embryogenic efficiency (%)	no. of germinated embryos	no of recovered plantlets
1	95.0 a	0.70 b	0.25 b	3 b	2 a
2	25.0 b	2.15 a	0.90 a	12 a	3 a
3	33.0 b	0.70 b	0.40 b	7 ab	3 a

3. 2. Effects of carbohydrate source

Carbon source had a profound affect on callus formation and growth in cvs. Regina (Table 2) and Reichensteiner (Table 3). In cv. Regina, sucrose achieved the highest callus formation followed by sucrose + mannitol, glucose and maltose respectively. Medium containing mannitol inhibited callus formation. In general, callus colour was yellow and changed gradually to light brown as time progressed. The best carbon source for callus formation in cv. Reichensteiner was sucrose while maltose was the worst. Sucrose was also significantly better than galactose and glucose. Carbon source also had a highly significant effect on somatic embryogenesis from anthers of both cultivars as somatic embryos appeared on sucrose- and glucose- supplemented media only.

Table 2. Effects of carbon source on callus formation and somatic embryogenesis in anthers of cv. Regina.

Carbon source	callus formation (%)	embryogenic explants (%)	embryogenic efficiency (%)	no. of germinated embryos	no of recovered plantlets
2% sucrose	89.0 a	6.0 a	5.1 a	50 a	12 a
2% sucrose + 2% mannitol	71.0 b	0.0 b	0.0 b	0 b	0 b
1.5% glucose	33.0 c	2.0 ab	1.4 ab	8 ab	5 ab
2% maltose	25.0 c	0.0 b	0.0 b	0 b	0 b
2% mannitol	0.0 d	0.0 b	0.0 b	0 b	0 b

In cv. Regina, embryogenic efficiency, germinated embryos and recovered plantlet numbers were higher in sucrose- than in glucose-supplemented media. However, in cv. Reichensteiner glucose was better than sucrose.

Table 3. Effects of carbon source on callus formation and somatic embryogenesis in anthers of cv. Reichensteiner

Carbon source	callus formation (%)	embryogenic explants (%)	embryogenic efficiency (%)	no. of germinated embryos	no of recovered plantlets
2% sucrose	50.0 a	2.0 ab	2.5 ab	10 ab	15 a
2% glucose	28.0 b	4.0 a	3.2 a	25 a	13 ab
2% galactose	36.0 b	0.0 b	0.0 b	0 b	0 b
2% maltose	9.0 c	0.0 b	0.0 b	0 b	0 b

3. 3. Effects of gelling agents

In comparing agar at 0.7% with Gelrite at 0.25%, both supported callus formation in cv. Reichensteiner during 4 weeks of culture (Table 4). No significant differences were observed in % callus formation, embryogenic explants, or embryogenic efficiency.

Table 4. Effects of gelling agents on callus formation and somatic embryogenesis in anthers of cv. Reichensteiner.

Gelling agent	callus formation (%)	embryogenic explants (%)	embryogenic efficiency (%)	no. of germinated embryos	no of recovered plantlets
0.7% agar	39.0 a	4.0 a	1.95 a	18 a	13 a
0.25% Gelrite	37.0 a	5.0 a	2.65 a	22 a	13 a

3. 4. Development of somatic embryos

Normal and abnormal somatic embryos were obtained at 42% and 58% for cv. Regina, 26% and 74% for cv. Reichensteiner respectively. Abnormalities of somatic embryos were manifested in the form of fused cotyledons and arrested growth. Normal plantlets were weaned and grew in the greenhouse and their appearance was normal.

4. Discussion

In this study two weeks of cold pretreatment increased the frequency of embryogenic explants, embryogenic efficiency and led to more germinated embryos with the cultivar Regina. The beneficial effect of cold pretreatment has been reported elsewhere (Rajasekaran and Mullins, 1979; Mauro et al., 1986).

Sucrose and glucose promoted somatic embryogenesis in cvs. Regina and Reichensteiner. Sucrose or glucose are widely used as carbon sources for plant cells grown in culture (Verma and Dougall, 1977). Murashige (1974), claimed that sucrose was most suitable in many cases, but that glucose was occasionally superior. Other carbohydrates may substitute for sucrose, or glucose, depending upon the species (Maretzki et al., 1974), e. g. galactose for citrus (Cabasson et al., 1995) and carrot (Verma and Dougall, 1977), fructose for pea (Loiseau et al., 1995) and maltose, fructose for alfalfa (Strickland et al., 1987). The inhibitory effect of fructose on callus formation in this study was also observed in carrot root cultures by Stehsel and Caplin (1969). In nature, carbohydrates are frequently transported within plant tissues as sucrose and thus plant tissue may have an inherently better capacity for uptake, transport and utilisation of sucrose (Eapen and George, 1993). In grape, most incidences of somatic embryogenesis reported occurred when sucrose was used as the sole carbon source (Emershad and Ramming, 1994; Mozsar and Sule, 1994; Perl et al., 1995).
No significant difference was found between the effects of agar and Gelrite at the concentrations tested and both yielded the same number of normal plantlets. The type of

gelling agent used has been found to influence callus formation and somatic embryogenesis in cucumber (Ladyman and Girard, 1992) and grape (Perl et al., 1995) cultures. Gelrite is also reported to promote shoot hyperhydricity (vitrification) in white ash (Bates et al., 1995) and the production of malformed somatic embryos in cucumber (Ladyman and Girard, 1992).

5. Conclusion

Somatic embryogenesis was induced in cvs. Regina and Reichensteiner on sucrose- and glucose-supplemented media. To enhance embryogenesis 2 weeks of cold pretreatment was beneficial, while agar and Gelrite were similar in their effects on embryo formation and development.

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