Vitis **52** (3), 137–140 (2013)

Use of amino acids for a highly efficient somatic embryogenesis in grapevine 'Crimson Seedless'

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Summary

Somatic embryogenesis influenced by growth regulators and amino acids was studied in *in vitro* leaves of grapevine 'Crimson Seedless'. *In vitro* leaves of the cultivar were collected from multiple shoot cultures maintained on Murashige and Skoog's (MS) basal medium supplemented with 9 µM N^6-benzyladenine (BA). Among the growth regulators used, BA at 4.5 µM induced higher embryogenic response producing more number of somatic embryos per explant. This response was increased with the addition of 5 µM naphthoxy-1-acetic acid (NOA) to ½ MS containing 4.5 µM BA. Further, supplementation of amino acids in the callus induction medium significantly improved the embryogenic response of *in vitro* leaves. The higher number of explants showing somatic embryo production (55.3 %) and higher number of somatic embryos per explant (15.5 per explant) were recorded with the supplementation of 5 mM phenylalanine to callus induction medium. Primary somatic embryos showed repetitive embryogenesis on ½ MS medium devoid of growth regulators. Plantlets derived from somatic embryos were transferred to soil-sand-peat mixture (1:1:1 v/v) and hardened plantlets were established in greenhouse with 90 % survival. This somatic embryogenesis system has been successfully used for *Agrobacterium*-mediated transformation studies in 'Crimson Seedless' in our laboratory. To our knowledge, this is the first report on the use of amino acids for the high efficient somatic embryogenesis in grapevine.

K e y w o r d s : Amino acids, 'Crimson Seedless', somatic embryogenesis, *Vitis vinifera* L.

Introduction

Somatic embryogenesis is the most utilized model system for plant totipotency and genetic transformation studies due to the high degree of competence for secondary somatic embryogenesis and plant regeneration. Nevertheless, in recent years somatic embryogenesis has been widely applied in breeding programs, and today it is the most suitable system for *in vitro* manipulation of grape genome (Martinelli and Gribaudo 2009). In grapevine, somatic embryogenesis system is often used for gene transfer studies, where whole somatic or zygotic embryos were used for co-cultivation with *Agrobacterium* followed by secondary embryogenesis. Occasionally, leaf tissues were also used for co-cultivation followed by somatic embryogenesis (Bornhoff *et al.* 2005).

'Crimson Seedless', a red table grape variety, originally developed in California, USA is a recent introduction to India and is favorably received due to the qualities more appealing to consumers such as crisp and firm berries. In order to meet the growing demand for its planting material and for improvement of the cultivar through genetic engineering, a highly efficient and reproducible regeneration system is needed. In earlier studies on 'Crimson Seedless', somatic embryos were induced from ovaries and immature anthers of the cultivar (Lopez-Perez *et al.* 2005). Addition of charcoal to the medium was found essential to obtain somatic embryos. They used this protocol for genetic transformation studies in 'Crimson Seedless' (Lopez-Perez *et al.* 2008). However, low germination and transformation efficiencies were recorded. Considering these, the present study was undertaken to develop a highly efficient and reproducible somatic embryogenesis system to be used for gene transfer studies in 'Crimson Seedless'.

Material and Methods

In vitro leaves derived from multiple shoot cultures (described in Nookaraju *et al.* 2008) of 'Crimson Seedless' were used as explants for the study. *In vitro* leaves measuring one cm^2 were cultured for callus induction in petri dishes (85 mm x 15 mm) with their abaxial surface in contact with the medium for callus induction. For initial screening of growth regulators, half-strength MS (½ MS) medium (Murashige and Skoog 1962) supplemented with different growth regulators singly were used for callus induction. Based on preliminary results, various auxins were supplemented in ½ MS containing 4.5 µM N^6-benzyladenine (BA) for further screening of growth regulator combinations. Influence of amino acids on somatic embryogenesis was studied using six amino acids at concentrations 2.5 and 5 µM (Table) supplemented in optimal callus induction medium (CIM), ½MS containing 4.5 µM BA and 5 µM naphthoxy-1-acetic acid (NOA).

In the present study, we used a two-step protocol for the induction of somatic embryos from *in vitro* leaves of 'Crimson Seedless'. *In vitro* leaf-derived calli were

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Table

Influence of amino acids on somatic embryogenesis from *in vitro* leaves of 'Crimson Seedless'

Amino acid (mM)	No. of explants cultured	% No. of explants showing embryogenesis	No. of embryos per explant ± SD
Control	36	30.56[c]	7.2 ± 1.3
Glutamine (2.5)	36	44.44[b]	6.7 ± 1.7
Glutamine (5.0)	36	44.44[b]	8.7 ± 2.0
L-Cystein (2.5)	36	41.67[b]	8.5 ± 1.5
L-Cystein (5.0)	36	47.22[b]	9.2 ± 1.3
Proline (2.5)	40	45.00[b]	11.7 ± 2.8
Proline (5.0)	40	45.00[b]	11.8 ± 1.0
Methionine (2.5)	40	35.00[c]	12.9 ± 0.7
Methionine (5.0)	40	30.00[c]	13.9 ± 3.0
Phenylalanine (2.5)	39	53.85[a]	14.6 ± 2.5
Phenylalanine (5.0)	38	55.26[a]	15.5 ± 2.7
Arginine (2.5)	38	42.11[b]	8.3 ± 1.7
Arginine (5.0)	39	46.15[b]	9.1 ± 2.7
SEM±		2.96	-
CD (p=0.01)		6.51	-
		**	-

Control: Pre-optimized callus induction medium (CIM, ½ MS + 4.5 µM BA + 5 µM NOA); ** Significant at 1% level. Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test (p = 0.01).

(Figure, a) maintained on callus induction medium for 4 months with regular subcultures at 4-weekly intervals, and they were subsequently transferred to hormone free ½ MS for somatic embryo induction. Observations on somatic embryogenesis were recorded after 60 d culture. Primary somatic embryos were cultured continuously on hormone free ½ MS for repetitive embryogenesis. For germination and conversion, mature cotyledonary stage somatic embryos were cultured on pre-optimized germination medium, Woody Plant Medium (WPM, Lloyd and McCown 1981) supplemented with 0.5 µM BA and 0.05 µM indole-3-butyric acid (IBA). Each treatment in all the experiments contained a minimum of 12 explants and each experiment was repeated three times. The data were analyzed using analysis of variance (ANOVA) and treatment means were compared by Duncan's multiple range test (Duncan 1955).

All the media were supplemented with 3 % (w/v) sucrose and pH of the media was adjusted to 5.8. All media were gelled with 0.65 % (w/v) agar. Cultures up to embryo induction and repetitive somatic embryogenesis were maintained under continuous dark, and shifted to 16 h photoperiod at a light intensity of 12.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 25 ± 1 °C for embryo germination and plantlet conversion. Somatic embryo-derived plantlets were transferred to plastic cups containing a mixture of soil, sand and coco-peat (1:1:1 v/v) and were hardened as described (Nookaraju *et al.* 2008). For histology studies, somatic embryos at various developmental stages were fixed in FAA (formaldehyde-glacial acetic acid-ethanol, 1:1:19) and dehydrated in ethanol - tertiary butyl alcohol series. Then the explants were embedded in paraffin wax and were sectioned (10 µm) with microtome (Leica RM 2155). The sections were stained by hematoxylin and eosin and observed under Axioplan microscope (Carl Zeiss, Germany).

Results and Discussion

The efficiency of callus induction and somatic embryogenesis from *in vitro* leaves of 'Crimson Seedless' varied with different growth regulators and amino acids used in the callus induction medium. Among the growth regulators, explants cultured on ½ MS supplemented with 4.5 µM BA for callus induction showed a higher somatic embryogenesis and this response was further increased with the addition of auxin (data not shown). Among the auxins, addition of 5 µM NOA to ½ MS containing 4.5 µM BA produced a higher embryogenic response (Figure, b).

An additional supplementation of this pre-optimized callus induction medium (CIM, ½ MS + 4.5 µM BA + 5 µM NOA) with amino acids enhanced the frequency of somatic embryogenesis with the exception of methionine (Table). Among all, explants cultured on CIM supplemented with 5.0 µM phenylalanine induced somatic embryogenesis in higher number (55 %) of explants with an average of 15.5 embryos induced per explant (Table). Further, the explants cultured on phenylalanine showed white compact to semi-compact callus with no visible signs of browning. Similar to our studies, amino acids were reported to improve embryogenesis from cultured anthers of *Triticum aestivum* (Indriantoro *et al.* 1999) and *O. sativa* (Guzmán and Zapata-Arias 2000). Somatic embryo production was promoted

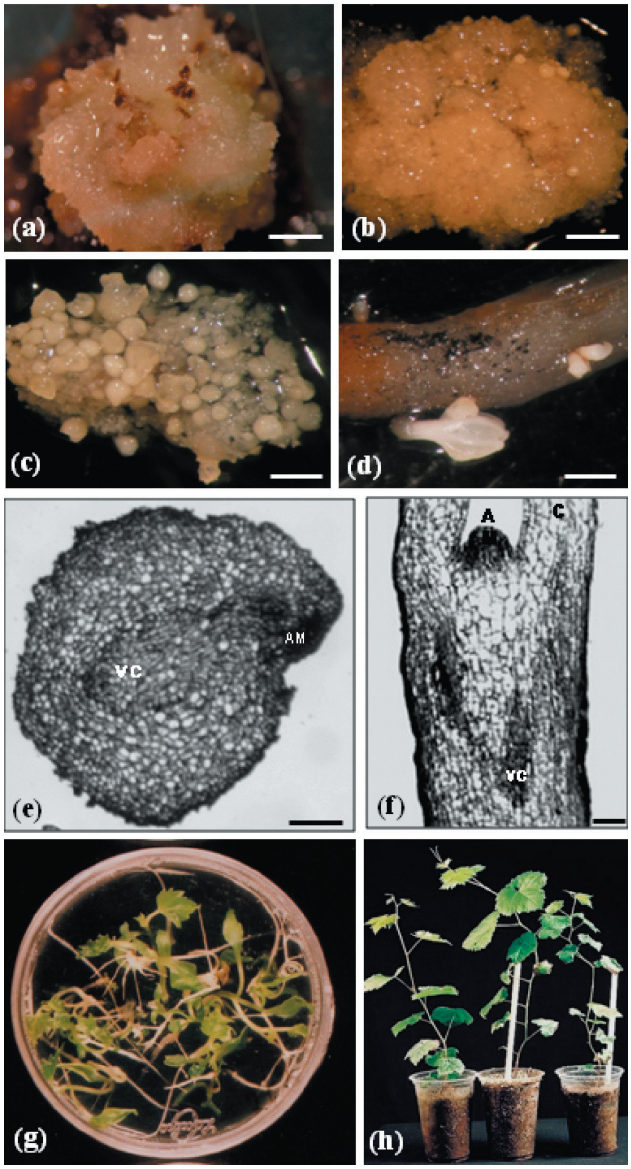

Figure: Somatic embryogenesis from *in vitro* leaves of 'Crimson Seedless'. Embryogenic callus (**a**) and somatic embryo induction from leaf-derived callus cultured on ½ MS + 4.5 µM BA and 5 µM NOA (**b**), proliferation of somatic embryos on ½ MS (**c**), secondary embryos from hypocotyls of germinated embryo (**d**), histology of globular (**e**) and cotyledonary stage (**f**) embryos showing cotyledons (C), apical meristem (AM) and vascular connections (VC), germinated embryos (**g**) and hardened plantlets (**h**). Bar = 2 mm (**a-d**) and 100 µm (**e-f**).

with the addition of amino acids in geranium (Murthy *et al.* 1996) and an addition of L-proline to the culture medium in soybean, green gram (Girija *et al.* 2000) and sugar beet (Moghaddam and Taha 2005). Similarly in *Cucumis sativus* L., addition of a combination of amino acids to callus induction medium had enhanced both somatic embryogenesis and plantlet regeneration (Kumar *et al.* 2003). In recent studies, addition of L-glutamine and L-proline had enhanced the embryo growth and development in *Gerbera* (Hasbulla *et al.* 2011). All these reports support our findings on enhancement of somatic embryogenesis by amino acids in grapevine. However, the exact mechanism by which amino acids exert their positive influence on somatic embryogenesis is still not clear. Amino acids are building blocks of proteins and they are known to play key roles in different plant metabolic processes via deamination and transamination reactions. The beneficial effect of amino acids on somatic embryo formation may be imputed to the enhancement of DNA amplification, increased cell division and growth.

Primary somatic embryos thus induced were sub cultured on fresh ½ MS for the induction of secondary embryos. Secondary embryos formed more frequently as clusters via callus phase (Figure, c) and at the radicle - hypocotyl transition zone of germinated embryos with or without intermittent callus (Figure, d). Histology of globular (Figure, e) and mature cotyledonary stage (Figure, f) embryos showed the presence of distinct apical meristem, bipolar vascular bundles and cotyledons. Mature cotyledonary stage somatic embryos showed 60 % germination on germination medium after 30 d culture. Germinated embryos grown to normal embryolings (Figure, g) were transferred to peat-sand-soil (1:1:1) mixture and then moved to greenhouse for hardening (Figure, h), which showed a 90 % survival.

Thus, present study demonstrates the high frequency somatic embryogenesis from *in vitro* leaves of grapevine 'Crimson Seedless' with the aid of growth regulators and amino acids supplemented in the callus induction medium. The protocol developed in our laboratory has been successfully utilized for *Agrobacterium*-mediated gene transfer studies in 'Crimson Seedless' for imparting downy mildew tolerance with high transformation frequency (Nookaraju and Agrawal 2012). To our knowledge, this is the first report on the use of amino acids for the highly efficient somatic embryogenesis in grapevine.

Financial support by the Department of Biotechnology (DBT), Govt. of India; Senior Research Fellowship (SRF) by the Council of Scientific and Industrial Research (CSIR), Govt. of India and supply of plant material of ‘Crimson Seedless’ by National Research Centre for Grapes, Pune, India are gratefully acknowledged.

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Received November 20, 2012