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# Synergistic effect of CPPU and benzyladenine on embryo rescue in six stenospermocarpic cultivars of grapevine

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## Summary

***In ovulo*/embryo culture technique has been used to recover hybrids from seedless grapevines. The present investigation was carried out to study the influence of pre-bloom sprays of N-(2-chloro-4-pyridyl)-N´-phenylurea (CPPU) - a synthetic cytokinin, and $N^6$-benzyladenine (BA) supplemented in culture media on embryo recovery in six stenospermocarpic grapevine cultivars. The results showed synergistic effect of CPPU and BA on embryo recovery. Though a CPPU spray alone increased embryo recovery in four out of six cultivars tested, the efficiency was enhanced several fold on culture of excised ovules on media supplemented with BA. The percentage of embryo recovery also depended on BA concentration and varied among six cultivars indicating a genotypic influence as well. Germination percentage of rescued embryos varied among the six cultivars and mostly corresponded with embryo recovery. Germinated embryos developed into normal plantlets. Present study demonstrates that spraying of panicles with CPPU and incorporation of BA in the ovule culture medium can enhance the embryo recovery in stenospermocarpic cultivars of grapevine.**

K e y w o r d s : Benzyladenine, CPPU, embryo rescue, seedless grapes.

A b b r e v i a t i o n s : BA = $N^6$-benzyladenine; CPPU = N-(2-chloro-4-pyridyl)-N´-phenylurea; WPM = Woody Plant Medium; ER = Emershad and Ramming.

## Introduction

Consumers all over the world increasingly prefer seedless table grapes. Hence grape improvement programmes have been aimed at developing new seedless varieties having better fruit quality, larger berry size and higher yields. Conventional hybridization to obtain seedless progenies using seeded cultivars as female parents has limited use due to a lower proportion of seedless progeny (Spiegel-Roy *et al*. 1990). In stenospermocarpic table grapes, fertilization takes place but embryo and/or endosperm development stops soon after anthesis, as a result seeds abort in different stages of growth depending on the cultivar (Bouquet and Davis 1989). Through *in ovulo* culture technique, it is possible to rescue such embryos before abortion, culture them and produce seedlings (Emershad and Ramming 1984, Gray *et al*. 1987, Spiegel-Roy *et al*. 1990, Bharathy *et al*. 2003). In breeding programmes earlier, seedless cultivars could only be used as pollen parents. However, with embryo rescue technique, it is possible to use seedless vines as female parents.

CPPU (N-(2-chloro-4-pyridyl)-N´-phenylurea) commonly known as forchlorfenuron), a synthetic cytokinin has significant physiological activity on many fruits including grapes. During the last decade, CPPU has been widely used in vineyards world over to change berry characteristics including larger berry size. Initial work on grapes performed by Dr. L. Nickell at Velsiocol Chemical Co., USA showed that size of 'Thompson Seedless' berries could be increased by 100% or more by application of 5-10 ppm CPPU at fruit set. Also he investigated interaction between CPPU and gibberellic acid (GA) on seedless grapes and found synergistic effects both on berry growth and maturity (Dokoozlian 2001). Present investigation aimed to study the influence of pre-bloom sprays of CPPU on embryo recovery and germination in six stenospermocarpic grapevine cultivars viz. 'Thompson Seedless', 'Crimson Seedless', '2A-Clone', 'Maroo Seedless', 'Kishmis Chernyi' and 'Mint'.

## Material and Methods

First spray of N-(2-chloro-4-pyridyl)-N´-phenylurea (CPPU) at 1 ppm was given to parrot green stage panicles *i.e.* about 10 d prior to flowering and second spray was given after 7 d to all the open pollinated vines of six stenospermocarpic grapevine cultivars ('Thompson Seedless', 'Crimson Seedless', '2A-Clone', 'Maroo Seedless', 'Kishmis Chernyi' and 'Mint'). CPPU powder was dissolved in an aliquot of Ethanol (94.5 %) and then made to the required volume with single distilled water. The vines have been maintained at the vineyard of National Research Centre for Grapes (NRCG), Pune. Immature berries (Figure, A) were collected at 55 d post anthesis from all six cultivars. After a pre-chilling treatment at 4 °C for one week, berries were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The berries were then submerged in 0.1 % fungicide solution (Bavistin™, BASF, India) for 1 h followed by 2-3 washes with sterile distilled water. Then the berries were treated with 0.1% (w/v) Mercuric chloride

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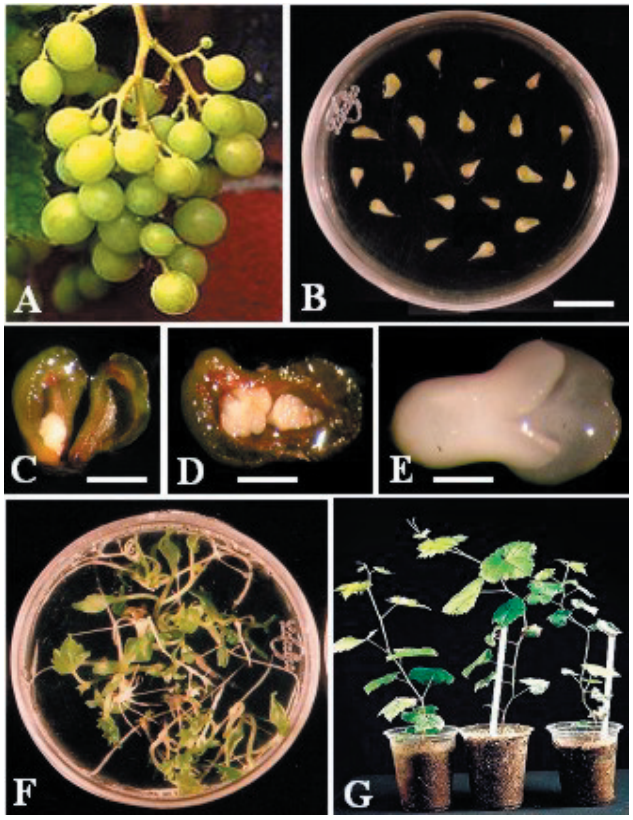


Figure: **A**: immature berries of 'Crimson Seedless', **B**: excised ovules (bar corresponds 6.4 mm), **C**: dissected ovule with one embryo (bar corresponds to 1.4 mm), **D**: dissected ovule with two embryos (bar corresponds to 1 mm), **E**: mature zygotic embryo (bar corresponds to 480 µm), **F**: germinated embryos, **G**: hardened plants of 'Crimson Seedless'.

for 10 min followed by several rinses with sterile distilled water in a laminar flow hood.

Method of ovule / embryo culture was followed as previously reported (Bharathy *et al.* 2003). Berries were blotted dry on sterile filter paper and ovules were excised from the berries aseptically. The excised ovules were cultured on ER (Emershad and Ramming 1984) medium supplemented with BA at different levels (0.44-22.22 µM) and sucrose (6 %) (Figure, B). After 60 d of culture, embryos were excised from ovules aseptically and cultured on Woody Plant Medium (Llyod and McCown 1981) supplemented with BA (0.89 µM) and sucrose (3 %). Activated charcoal (0.2 %) was added to all media gelled with agar (0.65 %). Observations on number of embryos recovered under each treatment were recorded at 60 d after inoculation of ovules and percent embryo germination was recorded after 30 d of embryo culture. Percentage of embryo recovery was calculated based on the number of embryos recovered from the total number of ovules dissected, and percentage of embryo germination was calculated based on the number of embryos recovered. Germinated embryos could be grown into seedlings (Figure, F), hardened and developed into plantlets (Figure, G) according to method described in our earlier report (Bharathy *et al*. 2003). A total of 2107 and 1093 berries from un-sprayed and sprayed bunches, respectively were used for the study from all the six cultivars with three replications. The experiment was repeated three times and data collected on percentage of embryo recovery was subjected to analysis of variance (ANOVA).

## Results and Discussion

In a preliminary experiment with 'Crimson Seedless' and '2A-Clone', it was found that berries at 55 d after flowering (DAF) resulted in the highest embryo recovery under control conditions (data not shown). Thus, in our CPPU spray experiments, berries were collected at 55 DAF.

In the control sets (ovules cultured on medium devoid of BA) with or without sprays, the percentage of embryo recovery among the six cultivars varied drastically (from 0.0 to 41) (Table). CPPU sprays had negative influence on embryo recovery in 'Thompson Seedless' and 'Kishmis Chernyi', since no embryo could be recovered in these two cultivars. In the remaining four cultivars, a drastic increase in embryo recovery was observed on CPPU sprays, though percentages varied greatly among the cultivars. The maximum percentage of embryo recovery viz. 10.33 (non-spray) and 41.00 (spray) was recorded in 'Mint' followed by 8.17 (non-spray), 15 (spray) in 'Maroo Seedless'. These results clearly demonstrate that, sprays of CPPU, independent of BA in the culture medium, increased embryo recovery dramatically in 'Crimson Seedless', '2A-clone', 'Maroo Seedless' and 'Mint'.

In most of the treatments ovules had single embryos (Figure, C) with the exceptions of two in a few cases (Figure, D). Excised embryos were globular or torpedo in shape (Fig. E). On comparing the results on embryo recovery between non-spray and spray (but ovules cultured on medium supplemented with a range of BA from 0.44-22.22 µM), it was observed that BA at all concentrations improved embryo recovery in control sets (non-spray) of all cultivars except 'Mint' and 'Kishmis Chernyi' (Table). In sets of 'Thompson Seedless' and 'Kishmis Cherrnyi' with CPPU sprays, inclusion of BA (0.89-8.88 µM) in the medium resulted in the embryo recovery, where the sets without BA in the medium did not show any embryo recovery indicating a positive role of BA in embryo development.

A synergistic effect of CPPU sprays and BA in culture medium on embryo recovery was observed in four out of six stenospermocarpic cultivars of grapevine. The percentage of embryo recovery depended on concentration of BA in the medium and differed significantly among the cultivars indicating possible role of genotypes as well (Table). The most spectacular difference in embryo recovery affected by sprays of CPPU was observed in cultivars '2A-clone', 'Maroo Seedless' and 'Mint'.

Like embryo recovery, varying response was observed with regard to germination of embryos. Among the six cultivars, the highest germination (93.9 %) was obtained in 'Crimson Seedless' with CPPU sprays (Table). Sprays of CPPU had positive influence on germination in 'Crimson Seedless', 'Maroo Seedless' and 'Mint', while the reverse was true for 'Thompson Seedless', '2A-clone' and 'Kishmis Chernyi'.

Cytokinins play an important role in stimulating both cell division and cell enlargement as well as delay of tis-

T a b l e

Influence of pre-bloom sprays of CPPU and supplement of BA in culture media on percent embryo recovery and percent embryo germination in six stenospermocarpic cultivars of grapevine

| BA Conc. (μM) | Percent embryo recovery* | | | | | | | | | | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Thompson Seedless | | Crimson Seedless | | 2A-Clone | | Maroo Seedless | | Mint | | Kishmis Chernyi | | |
| | Control | Spray | Control | Spray | Control | Spray | Control | Spray | Control | Spray | Control | Spray | |
| 0.00 | 1.41 | 0.00 | 2.00 | 3.50 | 1.50 | 3.80 | 8.17 | 15.00 | 10.33 | 41.00 | 3.99 | 0.00 | 7.56 |
| 0.44 | 3.89 | 0.00 | 5.87 | 8.34 | 10.00 | 12.00 | 25.00 | 26.30 | 15.00 | 50.33 | 0.00 | 0.00 | 13.06 |
| 0.89 | 2.78 | 1.67 | 8.33 | 9.40 | 25.67 | 43.33 | 23.60 | 41.33 | 25.67 | 61.00 | 11.67 | 10.00 | 22.04 |
| 2.22 | 3.33 | 1.33 | 6.00 | 7.92 | 30.00 | 18.89 | 21.30 | 35.67 | 23.33 | 43.33 | 10.67 | 3.00 | 17.06 |
| 4.44 | 3.89 | 0.03 | 4.22 | 7.33 | 16.00 | 21.67 | 21.30 | 26.33 | 18.00 | 42.33 | 0.00 | 4.60 | 13.81 |
| 8.88 | 3.89 | 0.13 | 3.17 | 6.38 | 10.67 | 29.57 | 27.90 | 31.33 | 8.00 | 39.33 | 3.99 | 6.67 | 14.25 |
| 22.2 | 3.33 | 0.00 | 2.00 | 4.66 | 11.00 | 15.67 | 31.70 | 31.67 | 7.00 | 31.67 | 10.67 | 10.67 | 13.34 |
| Mean | 3.22 | 0.45 | 4.51 | 6.79 | 15.00 | 20.70 | 22.71 | 29.66 | 15.33 | 44.14 | 5.86 | 4.99 | |

Percent embryo germination*

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 50.00 | 0.00 | 50.00 | 93.90 | 83.30 | 67.20 | 44.30 | 54.90 | 11.60 | 29.20 | 8.70 | 0.00 | |

Percent embryo recovery*

| | Cultivar (C) | CPPU Spray (S) | BA levels (B) | CXS | CXB | SXB | CXSXB |
|---|---|---|---|---|---|---|---|
| SEM | 0.27 | 0.16 | 0.34 | 0.37 | 0.84 | 0.48 | 1.19 |
| ± CD | 0.46 | 0.27 | 0.59 | 0.65 | 1.45 | 0.84 | 2.06 |
| | ** | ** | ** | ** | ** | ** | ** |

** Significant at 1% level; SEM = standard error of mean; CD = critical difference (p = 0.01).

* Percent embryo recovery was calculated based on number of embryos recovered from total number of ovules excised and percent embryo germination was calculated based on number of embryos recovered.

sue senescence and fruit ripening. It is reported that cytokinins show activity till 4 weeks after anthesis in developing seeds, disappear during fifth week and remains absent till ripening of berries (Pandey 1982). The pre-bloom sprays of CPPU that has cytokinin-like properties and inclusion of BA in the medium may have overcome the deficiency of cytokinins, which eventually led to better ovule and embryo development. Cytokinins are assumed to establish seeds as sink for assimilates for regulating cell division, initially in the ovary and subsequently in the meristem of the embryos hence are required for seed development (Atkins *et al*. 1998). Thus, it is conceivable that exogenous supply of CPPU in form of pre-bloom sprays and inclusion of BA in the medium during ovule / embryo culture may enhance sink strength of these organs and result in the higher embryo recovery.

Primary physiological effects of CPPU on grapevines involve the regulation of fruit set, berry growth and development. In earlier studies, CPPU has been shown to stimulate higher fruit yields in grape (Intrieri *et al*. 1992; Zabadal and Bukovac 2006), apple (Greene 1989) and cranberry (Devlin and Koszanski 1988). Also CPPU stimulated fruit set when applied at or just before flowering in kiwifruit (Patterson *et al*. 1993). Increased embryo development in grapevine influenced by addition of BA in the medium (Gray *et al*. 1990) and pre-bloom sprays (Bhar-

ATHY *et al*. 2003) has earlier been reported. However to best of our knowledge, this is the first report on combined effect of CPPU and BA on embryo recovery in grapevine. Present study demonstrates that spraying of panicles with CPPU (1 ppm) and incorporation of BA (0.89 µM) in the ovule culture medium can enhance the embryo recovery; however, preliminary tests are needed because of the varying response of different grapevine cultivars.

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