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PRODUCTION OF *Brugmansia* PLANTS FREE OF *Colombian datura virus* BY *in vitro* RIBAVIRIN CHEMOTHERAPY

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ABSTRACT

Brugmansia x *candida* Pers 'Creamsickle' plants produced by *in vitro* treatment with ribavirin, and no thermal therapy, remained polymerase chain reaction (PCR-) negative for *Columbian datura virus* (CDV) after one year. The plants were produced by establishing *B.* x *candida* 'Creamsickle' shoot cultures on autoclaved MS basal medium (Murashige and Skoog, 1962), sucrose 30 g/L, myo-inositol 100 mg/L, thiamine HCl 1 mg/L, pyridoxine HCl 1 mg/L, nicotinic acid 1 mg/L, glycine 2 mg/L, BAP 1.1 µM, pH 5.7, and bacteriological agar (USB Corporation, Cleveland, Ohio, USA) 9.0 g/L with 15 ml of medium per 25×100 mm flat-bottomed glass culture tubes with polypropylene caps (Magenta Corporation, Chicago, Illinois, USA). The cultures were maintained in a growth room illuminated by cool-white fluorescent lamps (26 µmol m^{-2} s^{-1}), constant 27°C, and a 16h photoperiod. Four weeks after initiation, the cultures were transferred to the same medium in polypropylene capped glass tubes except that the BAP concentration was reduced to 0.5 µM. *In vitro*-derived shoots were excised and further dissected to 3-6 mm in length before transferring onto the same medium containing ribavirin at 0, 50, 87.5, or 100 mg/L; these shoots were cultured for 30 days. The ribavirin treated shoots were then transferred onto the multiplication medium without ribavirin for one subculture before being rooted *in vitro* on the same MS basal medium except with one half strength MS nitrogen salts and 3 µM IAA for four weeks followed by greenhouse acclimatization. *In vitro*-derived plants that expressed no CDV symptoms and tested PCR-negative one year after transfer to the greenhouse were produced over the entire range of 50-100 mg/L ribavirin tested. A single line, $CS2^2B$, from these PCR-negative plants was selected for long-term assessment - this line remains symptom-free and enzyme-linked immunosorbent assay-negative after 6 years.

Keywords: *Brugmansia*, *Columbian datura virus*, virazole, micro propagation, plant tissue culture.

INTRODUCTION

Brugmansia is a popular and economically important ornamental plant that is susceptible to a number of viruses including *Colombian datura virus* (CDV; Kahn and Bartels, 1968). Infected plants may be symptomless if adequately watered and fertilized during vegetative growth (Preissel and Preissel, 2002), with symptoms ranging from faint chlorotic spots to severe mosaic and rugosity. A study to determine the incidence of CDV in *Brugmansia* plants obtained from Florida, Connecticut, Wisconsin, and California in the United States found that the virus was widespread (Chellemi *et al*., 2011).

Two methods commonly used to produce "virus-free" plants include thermal and chemotherapies applied singly or in combination, followed by *in vitro* shoot-tip or meristem culture (Awan *et al*., 2007; Fletcher *et al*., 1998; Gopal and Garg, 2011; Ohta *et al*., 2011; Sedlak *et al*., 2007). Virus-free means a specific virus cannot be detected. The specific combination of temperature, chemicals, and method of *in vitro* culture for producing virus-free plants is determined by the sensitivity of the virus and plant to the treatment. For example, a heat treatment may be effective for inactivating a virus, but may be an unsuitable treatment if the plant is sensitive to heat. The objective is to inactivate the virus but not kill the plant.

We report a strategy to produce CDV-negative *Brugmansia* plants that uses the antiviral agent ribavirin, (Virazole; 1-β-D-ribofuranosyl-1H-1, 2, 4-triazole-3-carboxamide) a synthetic nucleoside that inhibits many mammalian RNA viruses (Chang and Heel, 1981), on *in vitro* shoot cultures.

MATERIALS AND METHODS

Plant material and initiation of shoot cultures

Source plants for *in vitro* shoot tip cultures were CDV-infected greenhouse-grown mother stock plants of *Brugmansia* × *candida* 'Creamsickle'. Plants were grown and maintained using standard horticultural practices. Shoot cultures were initiated from lateral buds and shoot tips dissected to about 5 mm to 10 mm tall followed by a disinfestation protocol that began with 95% ethanol for 1 minute with agitation. After decanting the excess ethanol, the explants were placed into a solution of 0.525% sodium hypochlorite plus two drops of Tween-20 per 100 ml with agitation. After 15 minutes, the sodium hypochlorite solution was decanted and the explants were rinsed three times with sterile water. The lateral buds and shoot tips were further dissected to about 3 mm to 6 mm tall before explanting onto the culture initiation medium. The initiation medium was MS basal medium (Murashige and Skoog, 1962), sucrose 30 g/L, myo-inositol 100 mg/L, thiamine HCl 1 mg/L, pyridoxine HCl 1 mg/L, nicotinic acid 1 mg/L, glycine 2 mg/L, BA 1.1 µM, pH 5.7, and bacteriological agar (USB Corporation, Cleveland, Ohio, USA) 9.0 g/L. The medium was autoclaved in 500 ml volumes for 20 minutes at 121°C and then poured into previously autoclaved 25 × 100 mm flat-bottomed glass culture tubes with polypropylene caps (Magenta

Corporation, Chicago, Illinois, USA) at 15 ml of medium per tube. The culture tubes containing the liquid medium were cooled in a vertical position in polypropylene racks (Magenta Corporation). Four weeks after initiation the cultures were transferred to the same medium in polypropylene capped tubes except that the BA concentration was reduced to 0.5 µM. Thereafter, the multiplying clumps of precociously enhanced lateral buds were cultured onto the same medium in 77 × 77 × 77 mm polycarbonate vessels with polypropylene closures (Magenta Corporation, Chicago, IL, USA) containing 35 ml medium per vessel. The cultures were maintained in a growth room provided by cool-white fluorescent lamps (26 $\mu mol.m^{-2}.s^{-1}$), constant 27°C, and a 16-h photoperiod.

Ribavirin treatment

In vitro-derived shoots were excised and trimmed to 3-6 mm in length and then transferred onto shoot multiplication medium containing ribavirin at 0, 50, 87.5, or 100 mg/L; these shoots were cultured for 30 days. Shoot multiplication medium was MS salts (Murashige and Skoog, 1962), sucrose 30 g/L, myo-inositol 100 mg/L, thiamine HCl 1 mg/L, pyridoxine HCl 1 mg/L, nicotinic acid 1 mg/L, glycine 2 mg/L, BA 0.5 µM, pH 5.7, and agar 9.0 g/L. At least six culture tubes per ribavirin concentration were used, with twelve tubes for the 87.5 and 100 mg/L concentrations. Ribavirin treated shoots were then transferred onto shoot multiplication medium without ribavirin for 30 days. Shoots were excised and rooted in vitro on the same culture medium except with one half strength MS nitrogen salts and 3 µM IAA for four weeks. Rooted shoots were transferred to the greenhouse and acclimatized.

Serological and RT-PCR detection of *Colombian datura virus*

Two months after ribavirin treated plants were moved to the greenhouse, plants were analyzed for the presence of CDV by serological- and nucleic acid-based detection methods. For serological detection, enzyme-linked immunosorbent assay (ELISA) for potyviruses (Agdia Inc., Elkhart, IN) was used per the manufacturer's instructions. For reverse-transcription-polymerase chain reaction (RT-PCR) detection, a portion of the CDV NIb/coat protein (CP) genes was amplified using primers as previously described (Chellemi *et al*., 2011). One year after ribavirin treatment plants were moved to the greenhouse, plants were assayed by ELISA. A single ELISA-negative plant was selected. Six years after ribavirin-treated plants were moved to the greenhouse, the single ELISA-negative plant was tested by ELISA and RT-PCR.

Data analysis

Because all ribavirin concentrations resulted in CDV-negative plants, a Fisher's exact test was conducted on the data using the software Prism 5 (Graphpad Software, La Jolla, CA, USA).

RESULTS

Because preliminary thermotherapy experiments indicated that heat therapy was detrimental to *Brugmansia* chemotherapy using ribavirin was selected. Ribavirin-treated plants showed little phytotoxicity to ribavirin, even at the highest concentration (100 mg/L). Thirty-six *in vitro*-derived ribavirin-treated plants were transferred to a greenhouse and, two months after transfer were tested for the presence of CDV using ELISA and RT-PCR (Figure-1, Table-1).

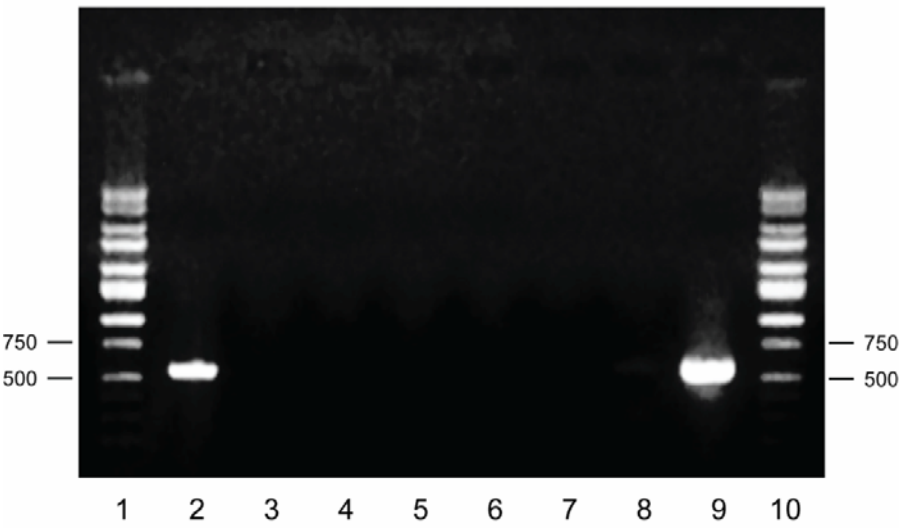

Figure-1. RT-PCR detection of CDV using primers - CDVv (5′-GGGAGAGCTCCTTACCTAGC-3′) and CDVvc 5′-CCATGTATGTTTGGTGATGTACC-3′) to amplify a 511 bp fragment of the NIb/CP genes (Chellemi *et al*., 2011). Lanes 1 and 10 –DNA size markers (~bp); Lanes 2 and 9 - 0 mg/L ribavirin, CDV-positive; Lane 3 - 0 mg/L ribavirin, CDV-negative; Lanes 4 and 5 - 50 mg/L ribavirin, CDV-negative; Lanes 6, 7, and 8 -100 mg/L ribavirin, CDV-negative.

Table-1. Two month old *in vitro*-derived greenhouse plants were tested for the presence of CDV by ELISA and RT-PCR analyses. The number in the parentheses is the number of plants that were retained for testing after one year. Grouping the data and comparing the number of CDV-positive and CDV-negative plants from shoot culture treated with no ribavirin (0 mg/L) or ribavirin (50, 87.5, and 100 mg/L) by Fisher's exact test resulted in a highly significant p value $\leq$ 0.0001.

Ribavirin mg/L	Number of plants treated	Number of CDV-positive plants	Number of CDV-negative plants
0	6	5 (5)	1 (1)
50	6	0	6 (6)
87.5	12	0	12 (6)
100	12	0	12 (8)

All plants treated with 50, 87.5, and 100 mg/L ribavirin tested negative. Five of the six 0 mg/L ribavirin (control) tested positive and one plant tested negative. Because of limitations on available greenhouse space, twenty-six plants were retained to be tested in one year - twenty-one CDV-negative plants and five CDV-positive plants.

One year after transfer to the greenhouse the twenty-six *in vitro*-derived ribavirin-treated plants were tested for the presence of CDV using ELISA. The CDV status of these plants was not changed from the prior two month test (Table-2); namely, the twenty-one 2-month CDV-negative plants remained ELISA-negative and the five CDV-positive plants remained ELISA-positive.

Table-2. One year old *in vitro*-derived greenhouse plants were tested for the presence of CDV by ELISA analysis. Grouping the data and comparing the number of CDV-positive and CDV-negative plants from shoot culture treated with no ribavirin (0 mg/L) or ribavirin (50, 87.5, and 100 mg/L) by Fisher's exact test resulted in a highly significant p value $\leq$ 0.0001.

Ribavirin mg/L	Total number of plants	Number of CDV-positive plants	Number of CDV-negative plants
0	6	5	1
50	6	0	6
87.5	6	0	6
100	8	0	8[a]

[a]A single plant was selected and maintained for 6 years in a greenhouse - it was CDV-negative by ELISA and PCR after 6 years.

CDV mottling symptoms were observed on CDV-positive plants but not on CDV-negative plants per a visual assessment (Figure-2).

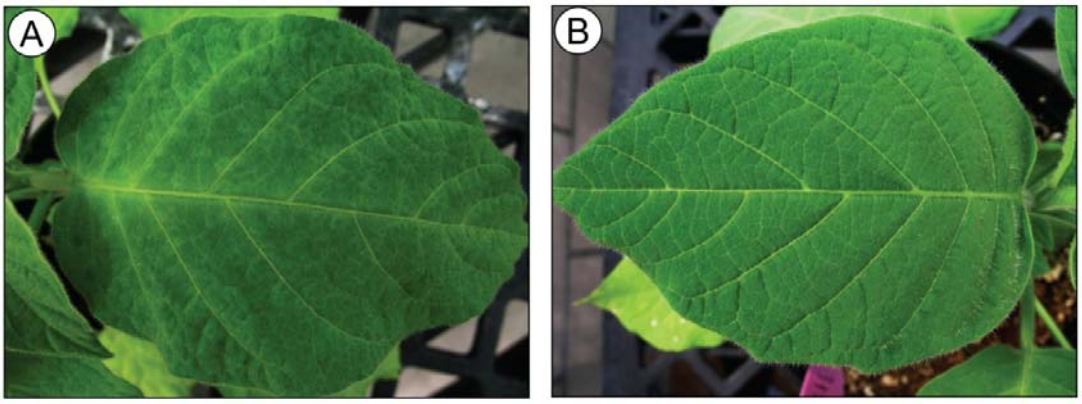

Figure-2. Visual assessment of CDV mottling symptoms on 12-month old ribavirin-treated plants. A) 0 mg/L ribavirin, ELISA CDV-positive, mottling symptoms; and B) 100 mg/L ribavirin, ELISA CDV-negative, no mottling symptoms.

A single CDV-negative plant, $CS2^2B$, derived from the 100 mg/L ribavirin treatment, was selected for long-term maintenance and observation. The single 100 mg/L ribavirin treated plant, $CS2^2B$, was tested by ELISA and RT-PCR six years after transfer to the greenhouse from *in vitro*. $CS2^2B$ tested CDV-negative by both ELISA and RT-PCR. Further, $CS2^2B$ had no visible mottling symptoms characteristic of CDV infection.

DISCUSSIONS

We developed a relatively simple plant tissue culture system to produce CDV-negative *Brugmansia* plants from CDV-positive *Brugmansia* plants. The procedure is built around a micropropagation system where shoot tips from stage 2 proliferating shoot cultures (Murashige, 1974) are grown for one culture cycle on proliferation medium containing ribavirin. The ribavirin-treated shoot tips are rooted *in vitro* and moved to *ex vitro* culture in the greenhouse. Compared to other plant species where ribavirin is typically used at concentrations ranging from 5-50 mg/L (Klein and Livingston, 1982; James *et al*., 1997; Awan *et al*., 2011; Gopal and Garg, 2011; Fletcher *et al*., 1998; Toussaint *et al*., 1993; Faccioli and Colalongo, 2002; Bittner *et al*., 1989; Faccioli and Colombarini, 1996; Greño *et al*., 1990; Verma *et al*., 2005; Sochacki, 2011; Weiland *et al*., 2004; Sedlak *et al*., 2011) and occasionally at 60-100 mg/L (Ohta *et al*., 2011; Singh *et al*., 2007), *Brugmansia* appears highly tolerant to ribavirin since little phytotoxicity was observed even at the highest tested concentration of 100 mg/L.

CDV-negative plants were readily produced from 50 mg/L ribavirin, the lowest level, other than 0 mg/L, tested. These results suggest that lower concentrations of ribavirin may be suitable for producing CDV-negative plants efficiently. The production of a single CDV-negative plant from 0 mg/L ribavirin indicates that the virus is not uniformly distributed in the plant. Thus, ribavirin is not essential for producing CDV-negative *Brugmansia* plants; it just makes the process considerably more efficient. These results also suggest that CDV is particularly sensitive to ribavirin.

MS salts (Murashige and Skoog, 1962) were used in this study for stage 1 shoot initiation and stage 2 shoot proliferations. However, though MS salts resulted in growth sufficient to produce CDV-negative plants, the system could be potentially improved by using the salt formulation recently developed for *Brugmansia in vitro* shoot growth (Niedz *et al*., 2011). This formulation provides a significant improvement in growth compared to MS salts and could be particularly useful for the micropropagation of *Brugmansia*.

With the results obtained from this study it is now possible to address the problem of widespread CDV infection of *Brugmansia* reported by Chellemi *et al*. (2011) and produce, maintain, and multiply CDV-negative *Brugmansia* plants commercially. First, *in vitro* shoot cultures would be established from desired cultivars. Second, shoot tips from these cultures would be treated with ribavirin as described in this study. Third, ribavirin-treated shoots would be rooted and established in a greenhouse. Fourth, established plants would be tested for the presence of CDV by ELISA and/or RT-PCR as described by Chellemi *et al*. (2011). Fifth, *in vitro* shoot cultures could be established from CDV-negative plants and maintained *ad infinitum* for conservation or as stock cultures for micropropagation of CDV-negative plants.

In conclusion, 1) *Brugmansia* is tolerant of high concentrations of ribavirin, relative to other plant species; 2) ribavirin is an effective antiviral compound for the *in vitro* production of CDV-negative *Brugmansia*; and 3) CDV is not uniformly distributed throughout *Brugmansia* plants.

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REFERENCES

Awan A.R., Mughal S.M., Iftikhar Y. and Khan H.Z. 2007. *In vitro* elimination of potato leaf roll polerovirus from potato varieties. Euro. J. Sci. Res. 18: 155-164.

Bittner H., Schenk G., Schuster G. and Kluge S. 1989. Elimination by chemotherapy of potato virus from potato plants grown in vitro. Potato Research. 32: 175-179.

Chang T.W. and Heel R.C. 1981. Ribavirin and inosiplex: a review of their present status in viral diseases. Drugs. 22: 111-128.

Chellemi D.O., Webster C.G., Baker C.A., Annamalai M., Achor D. and Adkins S. 2011. Widespread occurrence and low genetic diversity of *Colombian datura* virus in *Brugmansia* suggest an anthropogenic role in virus selection and spread. Plant Dis. 95: 755-761.

Faccioli G. and Colalongo M.C. 2002. Eradication of *Potato virus* Y and *Potato leafroll virus* by chemotherapy of infected potato stem cuttings. Phytopathol. Mediterr. 41: 76-78.

Faccioli G. and Colombarini A. 1996. Correlation of potato virus S and virus M contents of potato meristem tips with the percentage of virus-free plantlets produced in vitro. Potato Research. 39: 129-140.

Fletcher P.J., Fletcher J.D. and Lewthwaite S.L. 1998. *In vitro* elimination of onion yellow dwarf and shallot latent viruses in shallots (*Allium cepa* var. *ascalonicum* L.). New Zealand J. Crop Hort. Sci. 26: 23-26.

Gopal J., Garg I.D. 2011. An efficient protocol of chemo-cum-thermotherapy for elimination of potato (*Solanum tuberosum*) viruses by meristem-tip culture. Ind. J. Ag. Sci. 81: 544-549.

Greño V., Cambra M., Navarro L. and Durán-Vila N. 1990. Effect of antiviral chemicals on the development and virus content of citrus buds cultured in vitro. Scientia Hortic. 45: 75-87.

James D., Trytten P.A., Mackenzie D.J., Towers G.H.N. and French C.J. 1997. Elimination of apple stem grooving virus by chemotherapy and development of an immunocapture RT-PCR for rapid sensitive screening. Ann. Appl. Biol. 131: 459-470.

Kahn R.P. and Bartels R. 1968. The *Colombian datura* virus - a new virus in the Potato virus Y group. Phytopathology. 58: 587-592.

Klein R.E. and Livingston C.H. 1982. Eradication of potato virus X from potato by ribavirin treatment of cultured potato shoot tips. Am. Potato J. 59: 359-365.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Murashige T. 1974. Plant propagation through tissue cultures. Annu. Rev. Plant Physiol. 25: 135-166.

Niedz R.P., Hyndman S.E., Chellemi D.O. and Adkins S. 2011. *In vitro* shoot growth of *Brugmansia* × *candida* Pers. Physiol. Mol. Biol. Plants. 18: 69-78.

Ohta S., Kuniga T., Nishikawa F., Yamasaki A., Endo T., Iwanami T. and Yoshioka T. 2011. Evaluation of novel antiviral agents in the elimination of *Satsuma dwarf virus* (SDV) by semi-micrografting in *Citrus*. J. Japan. Soc. Hort. Sci. 80: 145-149.

Preissel U. and Preissel H.G. 2002. Brugmansia and datura: Angel's trumpets and thorn apples. Firefly Books, Buffalo.

Sedlak J., Paprstein F. and Talacko L. 2011. Elimination of *Apple stem pitting* virus from pear cultivars by *in vitro* chemotherapy. Acta Hort. 923: 111-115.

Singh B.R., Dubey V.K. and Aminuddin. 2007. Inhibition of mosaic disease of Gladiolus caused by Bean yellow mosaic- and Cucumber mosaic viruses by virazole. Scientia. Hortic. 114: 54-58.

Sochacki D. 2011. The use of ELISA in the micropropagation of virus-free *Narcissus*. Acta Hort. 886: 253-258.

Toussaint A., Kummert J., Maroquin C., Lebrun A. and Roggemans J. 1993. Use of VIRAZOLE® to eradicate *odontoglossum ringspot virus* from *in vitro* cultures of *Cymbidium* Sw. Plant Cell Tiss. Org. Cult. 32: 303-309.

Verma N., Ram R. and Zaidi A.A. 2005. *In vitro* production of Prunus necrotic ringspot virus-free begonias through chemo- and thermotherapy. Scientia Hortic. 103: 239-247.

Weiland C.M., Cantos M., Troncoso A. and Perez-Camacho F. 2004. Regeneration of virus-free plants by in vitro chemotherapy of GFLV (*grapevine fanleaf virus*) infected explants of Vitis vinifera L. cv 'Zalema'. Acta Hort. 652: 463-466.