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In vivo and *in vitro* Resistance Induction in Tobacco by *Boerhaavia diffusa* Systemic Resistance Inducing Protein and Transfer of Induced Resistance in *in vitro* Tobacco Plants

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Abstract: *Boerhavia diffusa* is a medicinally important plant. Root of *Boerhavia diffusa* contain basal proteins which show high virus inhibitory activity against plant viruses. Root extract of this plant induce strong systemic resistance in susceptible host plant. In the present study we found that the BD-SRIP induces the resistance against the TMV infection. The explant developed from the media containing the BD-SRIP extract show less degree of susceptibility against the viral infection.

Key words: NAA, IBA, MS, BD-SRIP

INTRODUCTION

Boerhavia diffusa (Family, Nyctaginaceae) is a herbal plant, commonly found in the Tropics in both dry and rainy seasons, mostly found in India, Nigeria and many other countries. The plant exhibits a somewhat periodic efficacy, with its maximum activity being noticed in the month of May (Rawat *et al*., 1994). The herbalists, however, use the aqueous leaf extract to treat diabetes in man. *B. diffusa* is used in traditional medicine for its anti-inflammatory, antibacterial and cardiotonic properties (Singh *et al*., 1994). It is used in the treatment of elephantiasis, night blindness and conceal ulcers. It is important naturally growing plant from which a systemic resistance inducing substance has been purified to homogeneity. This plant contains navel basic protein (30-34 kDa) capable of providing resistance/immunity to several susceptible hosts against commonly occurring viruses. The root of *B. diffusa* shows high virus inhibitory activity against plant viruses. Plant viruses reduce the yield and quality of horticultural, ornamental and vegetable crops. At present plant virus disease control by chemotherapeutic agents has met with limited success and alternate approach of managing these diseases is to induce the resistance/immunity in susceptible host plant, as no other effective methods of control are available. Treatments with aqueous extract from *B. diffusa* induce strong systemic resistance against viruses in several susceptible hosts (Verma *et al*., 1979a,b; Awasthi *et al*., 1994; Verma and Awasthi, 1980).

The purified virus inhibitory substance from root extract of *B. diffusa* L. has revealed that a basic protein (*Boerhavia diffusa* systemic resistance inducing protein BD-SRIP), endogenously occurring in this plant, is responsible for systemic resistance inducing activity (Verma and Awasthi, 1979; Verma *et al*., 1985). BD-SRIP stimulates the formation of a Virus Inactivating Agent (VIA) in the susceptible host. Tissue culture approach has been attempted to ensure availability of such naturally occurring anti viral substances through renewable stocks of *B. diffusa*.

The present study comprises of *in vitro* establishment of *Nicotiana tabacum* cv Samsum NN after immunizing/treated with the semi purified extract of roots of *B. diffusa*. The explants from treated host plants were regenerated and inoculated with virus TMV to confirm the transfer on induced resistance to the regenerated plants of Tobacco under *in vivo* and *in vitro* conditions.

MATERIALS AND METHODS

Plant materials and explants: The present study was carried out at University of Lucknow in the year 2000. The fresh explants of Tobacco were collected from the university campus to see their response under *in vitro* conditions. The explants were washed in liquid detergent Tween-20 (Himedia) and kept under running tap water until all traces of detergent were completely removed. Surface sterilization of explants were carried out in 0.1% aqueous solution of $HgCl_2$ for 2 min followed by rinse with autoclaved double distilled water (2-4 times). The surface sterilized explants were inoculated onto a sterile medium containing various hormonal combinations. MS

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(Murashige and Skoog, 1962) and other medium were used as basal medium throughout the experiment with different combinations and concentration of phytohormones, viz., auxins and cytokines, Sucrose (3%) mayoinositol (0.1%) were also added properly. MS0 and B50 were treated as control. The pH of the solution was checked and adjusted to 5.8 by adding 0.1 N NaOH and 0.1 N HCl (drop by drop) depending upon the solution, followed by the addition of 8% Agar Agar Type-1 (Himedia). The medium was autoclaved at 15l b pressure for 15 to 20 min at 121°C. After dispensing in tubes/vials culture were kept at a temperature of 25±3°C under cool white florescent tubes (200-3000 Lux) at 16-18 h photoperiod and relative humidity (50-69%).

Preparation of *Boerhaavia diffusa* root extract: The BD-SRIP was obtained from the freshly collected roots of *B. diffusa* growing in the university campus (Fig. 1). Fine powder was prepared after drying and grinding roots at 37°C in hot air oven. One hundred grams fine dry powder of root was soaked overnight at 4°C in (0.02 M) sodium acetate buffer (500 mL) with pH 5.2 (1: 5 w/v). 0.1% Mercaptoethanol was also added and squeezed through two folds of muslin cloth and the extract was centrifuged at 8000 rpm for 20 min. Supernatant was mixed with 60% Ammonium sulphate and kept overnight at 4°C. The precipitate was collected by low speed centrifugation and dissolved in minimum amount of sodium acetate buffer. This sample was passed through Sephadex G-25 column. Void volume elute from this column was used as virus inhibitor.

Desalting on sephadex G-25 column for obtaining BD-SRIP: Sephadex G-25 matrix (Pharmacia, India) was packed in a column (100×2.6 cm) to a height of 80 cm (void volume = 165 mL) and equilibrated with 20 mM sodium acetate buffer (pH 5.2), containing 0.02% sodium azide and 0.01% (v/v) 2-mercaptoethanol. Low molecular weight nonprotein component was also collected. Both the fractions were tested for their ability to induce resistance on the test host. The extract containing 30 kDa protein (Fig. 2) and possessing viral inhibitory activity was added to the mediumin different concentration or sprayed on test plants and subsequently explants were taken from these treated plants.

Virus inhibition assay method: Inhibition percentage was determined by local lesions assay using host reacting hypersensitivity (*N. tabacum* cv. Samsum NN) against TMV. The virus inoculum was prepared by crushing infected leaves of tobacco plant (N. *tabacum* cv. NP-31) with distilled water in a mortar. The pulp was squeezed through two folds of musclin cloth and the solution centrifuged at 6000 rpm for 15 min. The clear supernatant was used as virus inoculum after diluting it suitably with distilled water, so as to produce countable number of local lesions.

Fig. 1: *Boerhaavia diffusa* growing in field

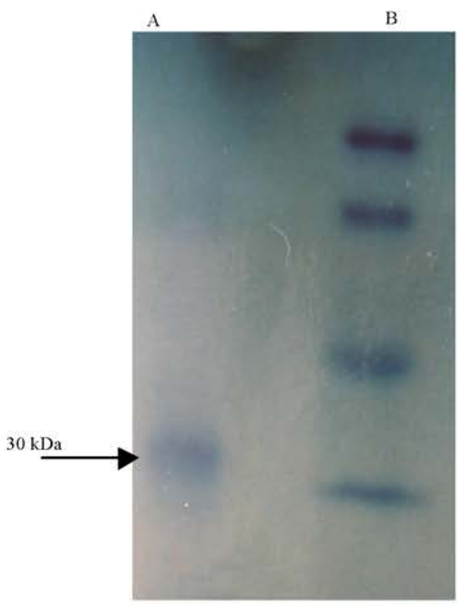

Fig. 2: SDS polyacrylamide gel electrophoresis of *Boerhaavia diffusa* root extract, Lane A-BD root extract, Lane B-Mol. et. Marker

Resistance induction in field grown/micropropagated plant: The BD-SRIP solution was applied/sprayed on two lower leaves of test plants. Control sets of plants were

sprayed with distilled water. After 2 h carborundum powder (600 mesh) was dusted on the upper surface of all inoculable leaves of a few treated and controlled plants and virus inoculated (TMV = 1: 200 w/v). The leaves were than rinsed with tap water. All the experiments were done in an insect free green house under natural light conditions. The local leisions appearing after 3-4 days were counted separately on treated and controlled set of plants. The remaining plants after 24 h of BD spray were used as explants and inoculated in MS medium containing no BD-SRIP and sterilized BD-SRIP (4%). Controlled plants (non sprayed) were also inoculated on MS medium containing no BD-SRIP.

Degree of inhibition calculated using the formula (C-T/C)×100
Where C = average no. of local lesions on control leaves.
T = average no. of local leaves on treated leaves

Raising of test plants: Test plants (*N. tabacum* cv. Samsun) were raised in an insect free green house in earthen pots containing sterilized compost and soil in the ratio of (1:1). Initially seeds were sown in bigger pots, when seedlings emerged out; they were separately transplanted in to smaller earthen pots.

Single node stem segment (a node with its axillary bud and small portion of internode on either side) measuring about 2 cm length were taken from test plant (N. *tabacum* cv. Samsun NN) and used as explants. The experimental material collected from field was surface sterilized in order to establish *in vitro*.

RESULTS AND DISCUSSION

The explant type and size shows great variation in the proliferation ratio of Tobacco plant. The best profile was seen in the buds as explant type. The size of explants which varies from 1-2 cm, large explant size increase the contamination ratio. The effect of sterilization agent was also prominent as increase in sterilizing % increased. The death rate increases the contamination.

Differentiation of axillary buds was influenced by the size of explant used. With very small explants, the survival rate was less, while using larger explant size the contamination was high.

It was noticed that by using smaller explant size, much lesser differentiation was obtained as compared to 1-2 cm explant size. Therefore, 1-2 cm explant size was used for bud proliferation.

The explants were subjected to 5 min treatment with 0.01 to 0.1% mercuric chloride solution. Sterilized plants were then inoculated on the surface of MS medium. In the optimum treatment by using 0.01% $HgCl_2$ all segments were obtained infection free, 20% sterilized explants turned brown showing toxicity. Eighty percent explants showed proliferation out of which 70% explants were viable.

Effect of explant position from natural field grown plants on bud proliferation: Nodal segments were taken from different positions of field grown plants and after sterilization were incubated on the MS-medium. The explants taken from upper portion having apical buds showed maximum bud proliferation in comparison to middle and lower portion, which showed poor proliferation. These were 100% viability in these explants and the growth response was fairly good.

Effect of growth hormones on axillary bud proliferation: The sterilized single node stem segments were transferred to MS medium with auxin, NAA+IBA (0.25+0.25 mg L^{-1}) with or without hormone. The explants in the medium with no growth hormone showed only 20% proliferation of axillary buds and poor growth. The explants grown in the medium containing NAA+IBA were showed 80% proliferation of buds as well as good growth. The cultures were subcultured after every 4 weeks. On subculturing the explants with out growth hormone in the medium showed better growth and rooting after 15 days of incubation. The number of roots increases from 4-12 cm and length increased 0.5-4.5 cm in the medium containing no growth hormone. Length of shoot increased from 2-7 cm and number of leaves also increased (Table 1).

Testing for induced resistance: The field induced (sprayed with BD-SRIP) Tobacco plants well tested for induced resistance. Control and treated plants challenged with TMV, lesions appeared on the inoculated leaves after 3 days of inoculation. These lesions were counted and degree of inhibition counted. Nighty two percent decrease in lesions number was observed in tobacco plants sprayed with BD-SRIP (Table 2).

Table 1: Effect of growth hormone on proliferation of different parameter of explants

Hormone (mg L^{-1})	Growth response	Length of shoots (cm)	Rooting (cm)	Proliferation (%)	Growth response
MS0	+++	1.5-7.0	0.5-4.5	20	+
NAA (0.5) +IBA (0.5)	++	0.5-3.0	0.3-1.5	80	++

+ Poor growth; ++ Good growth; +++ Better growth

Table 2: Test for induced resistance in field induced tobacco plants

Treatment	Plant	No. of lesions per leaf	Average No. of lesions	Percent reduction in lesion number
BD (1:5)	1	18+12 = 30/2 = 15		
	2	16+18 = 34/2 = 17	16	
	3	12+20 = 32/2 = 16		92
Control	1	180+244 = 424/2 = 212		
	2	148+210 = 358/2 = 179	201	
	3	206+218 = 424/2 = 212		

Degree of inhibition = C-T/C×100
= 201-16/201×100
= 92%

Table 3: Transfer of induced resistance to regenerants

Treatment	No. of lesions on different plants	Total No. of lesions on 6 leaves	Average/leaf	Reduction in lesion (%)
Explants from BD-SRIP	7+6=13/2			
sprayed plants and plants	8+6=14/2	34/6	5.7	74%
grown on MS medium	5+2=7/2			
containing BD-SRIP 4%				
Explants from control plants	12+19=31/2			
	18+15=33/2	130/6		
	33+33=66/2			

Transfer of induced resistance to regenerants: The BD-SRIP plants showing high degree of resistance were used as elite plants for obtaining explants. Explants were also taken from non sprayed control plants. These explants were inoculated on MS medium. The explants from treated plants were transferred to MS medium to which was added 4 and 100 mL of BD-SRIP after filter sterilization. The proliferated shoots were transferred for rooting. The rooted plants were subjected to hardening and when 4-5 inoculable leaves developed they were inoculated with TMV.

It was found that plants developing from treated explants grown on MS medium containing BD-SRIP were less susceptible to virus infection as compared to explants developing from control plants raised on MS medium. The result have shown that induced resistance induced resistance can be transferred to regenerants and explants grown on MS medium containing BD-SRIP showed high degree of resistance against virus infection. Thus, the results have shown that resistance plants can be developed utilizing BD-SRIP (Table 3).

CONCLUSIONS

The present study showed a procedure for transfer of induced resistance to regenerants. The resistance thus induced was not associated with any phytotoxic symptoms. This procedure would be suitable as an alternative for development of virus resistant plants. Since resistant varieties are not available in most of the crops. The only method perhaps for avoiding viral diseases would be to grow varieties with a high degree of viral tolerance or to increase the tolerance of these varieties by the use of systemic resistance inducing proteins such as present in *B. diffusa* root extract, so that yield of crop is not much affected by infection.

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REFERENCES

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

Rawat, A.K.S., S. Mehrotra, S.K. Tripathi and U. Shome, 1994. Hepatoprotective activity of *Punanarva*-a popular ethnomedicine. Ethnobiology in human welfare. Abstracts of 4th International Congress of Ethnobiology. Lucknow, Uttar Pradesh, India. 17th-21st November 1994:100.

Singh, M.K., S. Usha, S.C. Singh and U. Singh, 1994. Phytoecological investigations of *B. diffusa* Linn of Darbhanga district, Bihar. Neo-Botanical, 2: 61-64.

Verma, H.N. and L.P. Awasthi, 1979. Antiviral activity of *B. diffusa* root extract and the physical properties of the virus inhibitor. Can. J. Bot., 57: 926-932.

Verma, H.N., L.P. Awasthi and K. Mukerjee, 1979a. Isolation of the virus inhibitor from root extract *B. diffusa* inducing systemic resistance in plants. Can. J. Bot., 57: 1214-1217.

Verma, H.N., L.P. Awasthi and K. Mukerjee, 1979b. Induction of systemic resistance by antiviral plant extract in non- hypersensitive hosts. Z. Pflanezekr. Pflanzensch, 86: 735-740.

Verma, H.N. and L.P. Awasthi, 1980. Occurrence of a highly antiviral agent in plants treated with *B. diffusa* inhibited. Canad. J. Bot., 58: 2141-2144.

Verma, H.N., M.M.A.A. Khan and S.D. Dwivedi 1985. Biophysical properties of highly antiviral agents presents in *Pseudoranthemum atropurpurum* and *Bougainvillea spectabilis* extracts. Ind. J. Plant Pathol., 3: 13-20.