PRODUCTION OF INDIAN CITRUS RINGSPOT VIRUS FREE PLANTS OF KINNOW EMPLOYING CHEMOTHERAPY COUPLED WITH SHOOT TIP GRAFTING

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ABSTRACT

Indian citrus ringspot virus (ICRSV) is known to cause serious problem in Kinnow (Citrus nobilis Lour × C. deliciosa Tenora). This paper reports the elimination of ICRSV from Kinnow by chemotherapy coupled with shoot tip grafting under in vitro conditions. Nodal segments from infected mother plant (indexed by indirect ELISA and RT-PCR) were cultured on MS medium containing 2-iP (1mg/l) and malt extract (800 mg/l) along with different concentrations of five antiviral chemicals acycloguanosine, azidothymidine, 2,4-dioxohexahydro-1, 2,5-triazine (DHT), ribavirin and 2- thiouracil. Shoot tips of size 0.7 mm were excised from the sprouts of these nodal segments and grafted on to rough lemon (Citrus jambhiri) under aseptic conditions. The plantlets obtained from chemotherapy coupled with in vitro micrografting were indexed by indirect ELISA and RT-PCR after acclimatization. Maximum effect (37% virus elimination) was seen for ribavirin at 25 mg/l followed by 2-thiouracil at 25 mg/l (21.4%) and acyclguanosine at 25 mg/l (20.8%). Azidothymidine and DHT at the tested doses could not eliminate ICRSV. In the present study only those plants/plantlets were considered virus free, which showed negative reaction both with indirect ELISA and RT-PCR.

Keywords: Citrus, ICRSV (Indian citrus ringspot virus), Kinnow, Chemotherapy, Shoot tip grafting

INTRODUCTION

Mandarins, loose skinned Citrus fruits, constitute a commercially important group of fruit trees. Kinnow mandarin, a hybrid between King and Willow mandarins (Citrus nobilis Lour × C. deliciosa Tenora) is one of the most important fruit crops and its cultivation is considered to be a highly paying proposition in Punjab, India. In recent years, tremendous loss in yield and quality of this fruit crop has been observed due to various bacterial, fungal and viral diseases. A number of viruses are known to infect Kinnow trees and their consequences range from latent infection with little apparent effect on the host to its death. Among different viruses known to infect Kinnow, Indian citrus ringspot virus (ICRSV) is widely distributed throughout the country limiting the production of quality fruits [6, 44, 45]. The disease was first described by Wallace and Drake [54] from California and has since been reported from many areas worldwide. The leaves of infected plants exhibit typical chlorotic rings of variable diameter and drop prematurely. Several affected trees show dieback and decline type of symptoms and thus become less productive. A decrease in fruit weight, number, size and juice content of infected trees has also been observed [6, 25, 45, 46]. During a survey conducted in major Kinnow growing zones of Northern states of India, the percent incidence of the disease was found to range from 66% to 91.33% [39].

Kinnow is propagated vegetatively by grafting, therefore use of infected budwood acts as the main cause of spread of viral diseases. This necessitates the production of certified healthy virus free propagation material for establishing new orchards. A number of scientists have tried to explore the use of different antiviral compounds to obtain virus free plants from infected cultivars. Some of the commonly used antiviral chemicals for elimination of different plant viruses include acycloguanosine (Acyclovir), azidothymidine (Zidovudine), 2,4-dioxohexahydro-1,2,5-triazine (DHT), ribavirin (Virazole) and 2- thiouracil etc. [12, 14, 17, 23, 29, 32, 50, 51]. Literature survey has revealed that no attempt has been made to study the inhibitory effect of any antiviral chemical against ICRSV especially in Kinnow. The present study was planned to investigate the effect of different antiviral chemicals like acycloguanosine, azidothymidine, 2,4-dioxohexahydro-1, 2,5-triazine (DHT), ribavirin and 2- thiouracil coupled with in vitro shoot tip grafting for the elimination of ICRSV from Kinnow. The objective of this study was to combine beneficial effects of shoot tip grafting with chemotherapy to eliminate ICRSV from Kinnow.

MATERIALS AND METHODS

Selection of the mother plant

Kinnow plant growing at Government Nursery, Department of Horticulture (Punjab), Amritsar, India, naturally infected with Indian citrus ringspot virus (ICRSV) (as confirmed by indirect ELISA and RT-PCR), was used as a source of explants for raising in vitro cultures.

Virus detection

Indirect ELISA and RT-PCR were performed on leaves from infected field tree used as a source of explant and leaves from young acclimatized plantlets produced through chemotherapy coupled with in vitro shoot tip grafting. The procedures adopted for indirect ELISA and RT-PCR have been described in our earlier reports (42,43).

Chemotherapy

Chemotherapy was applied during nodal culture. The protocol and media requirements for nodal cultures have already been reported by us [33]. The nodal segments from ICRSV infected Kinnow plants, after proper sterilization, were inoculated on to MS medium containing 2-iP (1mg/l) and ME (800mg/l) along with various concentrations (5-25 mg/l) of antiviral chemicals viz. acycloguanosine, azidothymidine, DHT, ribavirin and2-thiouracil. The antiviral chemicals were filter sterilized before incorporation into autoclaved medium. The cultures were incubated in culture room maintained at 25±2ºC and 70% relative humidity under 40µmole/m^2/second cool white fluorescent light for 16 hours daily. Shoot tips excised form these nodal sprouts were used as scion and micrografted on to rough lemon in vitro. For micrografting, rootstock seedlings were raised by germinating seeds of C. jambhiri in culture tubes containing MS medium solidified with 1% bactoagar and maintained at a temperature 25 ± 2°C in continuous darkness. Two-week-old rootstock seedlings were removed from the culture tubes and decapitated under aseptic conditions to remove all the leaves. The roots were cut to a length of 4-6 cm; the cotyledons and their axillary buds were also removed. In a preliminary study, different sizes of shoot tips viz. 0.5 mm, 0.6 mm, 0.7 mm, 0.8 mm and 0.9 mm were used for shoot tip grafting. It was ascertained that shoot tips of size 0.7 mm gave best response and this size was used for further experimentation. For grafting, a vertical slit was made on the central part of the rootstock, and the scion base cut in a V shape was fitted into the slit. Grafted plantlets were aseptically cultured in liquid nutrient medium. A folded filter paper supportive platform perforated in the center for insertion of the root portion of the grafted rootstock was

placed in liquid MS basal medium lacking plant growth regulators. The cultures were maintained at 25±2ºC with a luminous intensity of 40µmole/m²/second daily for 16 hours. After five to six weeks of micrografting, plantlets with scion having at least two expanded leaves were transferred to autoclaved plastic pots containing a mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. High humidity conditions were maintained around grafted plantlets by covering them with clean transparent polythene bags having small holes for air circulation. After 8-10 days, the plantlets were exposed by removing polythene bags for 30-40 minutes. The exposure time was increased gradually for each day and after another 8-10 days, the bags were removed completely and the plantlets were transferred to the earthen pots containing garden soil only. After proper hardening, the plants were then transferred to the field.

Experimental design

To study the effect of different concentrations of various antiviral chemicals, three experiments were performed, each consisting of 24 cultures. The data obtained was statistically analyzed using SPSS for windows standard version (Release 7.5.1) for General Linear Model - General factorial. In order to estimate homogenous subsets for various treatments Post Hoc Test- Tukeys HSD at level of significance $\alpha = 0.05$ was applied.

RESULTS

Virus indexing

Virus indexing of the parent plant was done by employing indirect ELISA and RT-PCR. The latter showed an amplification of 539 bp fragment of partial coat protein gene indicating the presence of ICRSV. This plant was also found positive for ICRSV using indirect ELISA. The plantlets raised in vitro through chemotherapy coupled with shoot tip grafting were also tested for the presence/ absence of ICRSV using indirect ELISA and RT-PCR. Some of these plantlets were found negative for ICRSV (Table 1).

Chemotherapy

The effects of different concentrations of antiviral chemicals viz. acycloguanosine, azidothymidine, 2,4-dioxohexahydro-1, 2,5-triazine (DHT), ribavirin and 2-thiouracil on percent shoot formation from nodal buds, grafting success rate and elimination of ICRSV form the successful grafts are presented in Table 1. Maximum (37%) virus free plants were obtained with ribavirin at 25 mg/l, followed by 2- thiouracil at 25 mg/l (21.4 %) and acycloguanosine at 25 mg/l (20.8%). With azidothymidine and DHT, no virus elimination was observed (Table 1). As can be seen form Table 1, at highest concentration of azidothymidine tested, results with indirect ELISA have shown virus elimination in 11.1% of micrografts but later RT-PCR analysis of these plantlets has shown them to be positive for ICRSV. Besides elimination of ICRSV, all the antiviral chemicals showed phytotoxicity (Tables 1). A significant decrease in percent shoot formation and grafting success rate was observed with increase in concentration of the antiviral chemicals and this phenomenon was common to all the antiviral chemicals used in this study. Maximum phytotoxicity was observed with 2- thiouracil at 25 mg/l where shoot formation and grafting success were 19.44% and 12.5% respectively.

DISCUSSION

Enzyme Linked Immunosorbent Assay (ELISA) is routinely used for the detection of plant viruses [1, 34, 49]. It has been demonstrated that ELISA lacks the sensitivity required for detection of the viruses, which occur in low concentrations in plant tissues. In an attempt to overcome this problem, RT-PCR has been widely used by various workers for detection of plant viruses [27, 29, 51]. In the present study also, RT-PCR was found to be more sensitive than ELISA as it could detect ICRSV in some of the ELISA negative plantlets (Table 1).

This work shows that three (acycloguanosine, ribavirin and 2- thiouracil) out of the five antiviral chemicals tested can be used in combination with shoot tip grafting for elimination of ICRSV from Kinnow under in vitro conditions. Acycloguanosine at its higher concentration tested i.e. 25 mg/l was effective in elimination of ICRSV from Kinnow. Caner et al. [7] showed its inhibitory effect on Bean golden mosaic virus infecting Phaseolus lunatus when sprayed on the pre inoculated plants. Similarly, Ram et al. [32] were successful in eliminating Chrysanthemum B carlavirus with the use of acycloguanosine. On the contrary, antiviral effects of acycloguanosine could not be observed against Ornithogalum mosaic potyvirus [50] and Potato virus S [30]. The efficiency of ribavirin in the elimination of plant viruses is well documented in literature. It has been used against several plant viruses as a viricide such as Cymbidium mosaic virus and Odontoglossum ringspot virus [2, 24, 48, 53], Peanut mottle virus [9], Prunus necrotic ringspot virus [47], Potato virus M, S, X and Y [17, 29]. In the present study also, ribavirin was most efficient in elimination of ICRSV as compared to other antivirals tested. 2-thiouracil has also been reported to be effective in controlling plant viruses such as Apple mosaic virus [10], Citrus tristeza and Citrus infectious variegation ilarvirus [16], Cowpea banding mosaic virus [38], Lily symptomless, Tulip

Table 1: Effect of different concentrations of acycloguanosine, azidothymidine, DHT, ribavirin

Antiviral chemical	Concentration (mg/l)	Percent shoot formation (Mean ± SE)	Percent grafting success rate (Mean ± SE)	Percent virus elimination ELISA	Percent virus elimination RT- PCR
Acycloguanosine					
	Control	91.67 ± 0.00 [a]	54.17 ± 2.405 [a]	0.00	0.00
	5	70.83 ± 0.00 [b]	51.39 ± 1.39 [a]	0.00	0.00
	10	65.28 ± 2.78 [b]	40.28 ± 1.39 [b]	0.00	0.00
	15	45.83 ± 2.40 [c]	30.56 ± 1.39 [c]	0.00	0.00
	20	27.78 ± 2.78 [d]	22.22 ± 2.78 [cd]	15.38	0.00
	25	20.84 ± 4.17 [d]	15.28 ± 2.78 [d]	25.00	20.83
Azidothymidine					
	Control	91.67 ± 0.00 [a]	55.56 ± 1.39 [a]	0.00	0.00
	5	72.22 ± 2.78 [b]	51.39 ± 2.78 [ab]	0.00	0.00
	10	65.28 ± 1.39 [b]	41.67 ± 2.40 [bc]	0.00	0.00
	15	45.83 ± 2.405 [c]	33.33 ± 2.40 [cd]	0.00	0.00
	20	29.17 ± 2.40 [d]	22.22 ± 3.67 [de]	0.00	0.00
	25	22.22 ± 3.67 [d]	16.67 ± 2.40 [e]	11.11	0.00
DHT					
	Control	91.67 ± 0.00 [a]	55.56 ± 1.39 [a]	0.00	0.00
	5	75.00 ± 2.41 [b]	54.17 ± 2.40 [a]	0.00	0.00
	10	66.67 ± 2.40 [b]	44.45 ± 2.78 [ab]	0.00	0.00
	15	48.61 ± 3.67 [c]	36.11 ± 2.78 [bc]	0.00	0.00
	20	31.94 ± 3.67 [d]	25.00 ± 2.41 [cd]	0.00	0.00
	25	23.61 ± 5.01 [d]	18.06 ± 3.64 [d]	0.00	0.00
Ribavirin					
	Control	91.67 ± 0.00 [a]	55.56 ± 1.39 [a]	0.00	0.00
	5	72.22 ± 3.67 [b]	52.78 ± 1.39 [ab]	0.00	0.00
	10	66.67 ± 2.40 [b]	43.06 ± 3.67 [bc]	0.00	0.00
	15	47.22 ± 1.39 [c]	34.72 ± 3.67 [cd]	10.53	0.00
	20	30.55 ± 2.78 [d]	23.61 ± 1.39 [de]	23.08	15.38
	25	23.61 ± 1.39 [d]	16.67 ± 2.40 [e]	44.44	37.04
2- thiouracil					
	Control	91.67 ± 0.00 [a]	55.56 ± 1.39 [a]	0.00	0.00
	5	61.11 ± 2.78 [b]	50.00 ± 2.41 [ab]	0.00	0.00
	10	51.39 ± 1.39 [bc]	40.28 ± 1.39 [bc]	0.00	0.00
	15	38.89 ± 1.39 [cd]	29.17 ± 2.40 [cd]	0.00	0.00
	20	26.39 ± 3.67 [de]	20.83 ± 2.40 [de]	18.18	0.00
	25	19.44 ± 3.67 [e]	12.50 ± 4.17 [e]	28.57	21.43

Data shown are Mean ± SE of three experiments, each experiment consisted of 24 cultures
Means followed by the same letter are not significantly different from each other (General factorial – Tukey's HSD at α=0.05).

breaking virus-L and Cucumber mosaic virus [52], Mosaic virus of Vigna sinensis Endl. [18], Potato S carlavirus [11], Turnip yellow mosaic virus [31], Prunus necrotic ringspot virus [51] and Chrysanthemum B carlavirus [32]. In the present study also, ICRSV free Kinnow plants were produced with 2- thiouracil at 25 mg/l. Azidothymidine is a synthetic pyrimidine analogue and is known to inhibit animal and mammal retroviruses but not much work has been done on plant viruses. Ram et al. [32] for the first time were successful in eliminating Chrysanthemum B carlavirus using azidothymidine. However, in our study ICRSV elimination could not be achieved with azidothymidine even at its highest concentration tested i.e. 25mg/l. DHT has been reported to be an effective antiviral chemical for controlling plant viruses such as Potato virus S [3, 5], Potato virus X [4, 35, 36], Prunus dwarf virus, prune dwarf ilarvirus and prunus necrotic ringspot virus [20], Prunus necrotic ringspot virus and Apple chlorotic leaf virus [14]. However, in our study ICRSV elimination could not be achieved with DHT even at its highest concentration tested. This may be due to higher concentrations (100-400 mg/l) of DHT used by other workers for elimination of the viruses or due to difference in the virus under study. Due to significant phytotoxicity showed by acycloguanosine, azidothymidine and DHT, further higher concentrations were not tried in the present study. Phytotoxicity of the antiviral chemicals is also reported by many workers [14, 17, 19, 41]. The efficiency of antiviral chemicals as inhibitory agents of virus multiplication is said to be linked to their concentration, host plant considered, type of tissue being treated and to the type of virus [48]. However their efficiency also depends upon the stage of development of the tissue under consideration eg. ribavirin inhibits the replication of TMV in tabacco leaf cells (in situ) when treatment was started straight after inoculation, where as it has no effect on non differentiated calli infected with the same virus [26]. The biochemical events leading to virus eradication by chemicals are not clearly understood. The antiviral chemicals block virus replication and although existing virus might remain in the original stem sections, the new outgrowths would be virus free or contain only very low amounts of the virus [41]. Ribavirin may be active in its triphosphate form, which inhibits the 5′ capping of viral RNAs [13]. Schuster and Huber [37] concluded that both ribavirin and DHT inhibit potato virus X replication at the early stage by impairing synthesis of RNA- dependent RNA polymerase and at a later stage by impairing synthesis of the coat protein. Shepard [40] suggested a similar explanation to account for the fact that Potato virus X infected protoplasts of Nicotiana tabacum treated with ribavirin remained infected, where as embryonic shoots, differentiated from protoplasts derived callus were virus free.

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