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Preliminary screening of citrus hybrids for identifying tolerance to *Phytophthora nicotianae*

Harsimran Singh, Anita Arora and Krishan Kumar[1*]

Department of Fruit Science, Punjab Agricultural University, Ludhiana 141004, Punjab, India

ABSTRACT

***Phytophthora* is one of the most devastating pathogens affecting citrus production. Breeding resistant genotypes is a durable strategy to manage this pathogen. In this study, *in vitro* screening of 31 citrus hybrids was done against *Phytophthora nicotianae* using detached leaf assay. The hybrids tested were developed in two-way crosses between Rangpur lime (RL, *Phytophthora* sensitive) and sour orange (SO, *Phytophthora* tolerant). The relative *Phytophthora* sensitivity was determined from the necrotic lesion area after three days of pathogen inoculation. The parents and hybrids could be differentiated into five resistance classes on the basis of necrotic lesion size and area. Among the 18 SO × RL (SR) hybrids, three hybrids *viz.* SR 3, SR 10 and SR 19 exhibited the least leaf necrotic length (2.8-3.7 mm), breadth (3.2-3.9 mm) and area (9.2-15.0 mm^2), indicating a highly resistant reaction. From the 13 RL × SO (RS) hybrids, RS 6 and RS 22 showed highly resistant reaction by virtue of the least necrotic area (12.7-17.9 mm^2). Twelve SR and four RS hybrids produced necrotic lesions of >216.8 mm^2 area and were rated as highly susceptible in this assay. The parents- Rangpur lime and sour orange recorded highly susceptible and resistant reaction, respectively, which authenticated the results of the leaf assay. The five highly resistant hybrids identified in this study after *ex vitro* validation of *Phytophthora* tolerance should prove helpful in commercial citriculture.**

Key words: Citrus, Hybrids, Leaf inoculation, Necrotic lesions, *Phytophthora,* Tolerance.

INTRODUCTION

Citrus fruits are one of the most widely cultivated fruit crops in the world. These fruits are a rich source of vitamins, *viz*. ascorbic acid (vitamin C), folic acid (vitamin B9), thiamine (vitamin B1), niacin (vitamin B3), pyridoxine (vitamin B6), riboflavin (vitamin B2), pantothenic acid (vitamin B5) and minerals like potassium, calcium, phosphorus, magnesium, and copper. These also contain many healthy phytochemicals like carotenoids, flavonoids and limonoids, which prevent the inception of various chronic diseases (Liu *et al.*, 13). Various phytopathogens cause severe economic losses to citrus (Chen *et al.*, 5*;* Aboutorabi, 1). Among these, *Phytophthora,* an oomycetous fungus is considered to be the most notorious pathogen as it causes damage through diseases like damping off in nurseries, and foot rot, root rot, gummosis, leaf blight and brown rot of the fruits in orchards (Savita and Nagpal, 17). Globally, twelve species of *Phytophthora* are reported to be pathogenic on citrus, *viz*. *P. nicotianae*, *P. citrophthora*, *P. palmivora*, *P. boehmeriae*, *P. cactorum*, *P. capsici*, *P. citricola*, *P. cinnamomi*, *P. dreschleri*, *P. hibernalis*, *P. megasperma* and *P. syringae*. Out of these, *P. nicotianae* (syn. *P. parasitica*) is the most devastating and predominant species reported globally (Das *et al.*, 6; Panabieres *et al.*, 15). Direct losses afflicted due to *Phytophthora* are difficult to estimate, but an estimated 3-6% annual yield losses are reported in Florida citrus (Graham and Menge, 9). It is causing annual death of more than 20% citrus trees in central India and is considered the prime reason of lower tree productivity and tree decline in north India (Naqvi, 14). Breeding for resistance is viewed as an environment-friendly approach to managing this pathogen, as intensive use of chemicals may cause environmental and health hazards (Lima *et al.*, 12). Many citrus genotypes like trifoliate orange (*Poncirus trifoliata* L.), Swingle citrumelo [*Citrus paradisi* × *P. trifoliata*] (Graham, 8), sour orange (*C. aurantium* L.) (Ajengui *et al.*, 2) exhibit tolerance to *Phytophthora.* In India, rough lemon (*C. jambhiri* L.) is the principal citrus rootstock, which is susceptible to *Phytophthora* and soil salinity. To overcome these limitations, we have developed new hybrids between genotypes, Rangpur lime (*C. limonia* Osbeck) (tolerant to soil salinity) and sour orange (tolerant to *Phytophthora*). These hybrids have to be evaluated for their relative tolerance against these two stresses. *Phytophthora* can attack different parts of the plant, which also provides opportunities for screening using different plant tissues (Rajput *et al.*, 16). The rhizospheric inoculation of plants with pathogen inoculum is considered a reliable method

*Corresponding author: kkshorti@pau.edu

[1]Dr.JC Bakhshi Regional Research Station, Abohar 152116, Punjab, India

for assessing the *Phytophthora* tolerance. However, this method takes a longer time to produce visible symptoms (Twizeyimana *et al.*, 18). Alternatively, disease tolerance can also be judged *in vitro* by artificially inoculating the plant tissues. It is an easier and faster method of screening (Twizeyimana *et al.*, 18), where relative pathogen sensitivity can be determined from the area of necrotic lesions (Denman and Sadie, 7). In this study, we assessed the *Phytophthora* tolerance of new citrus hybrids using *in vitro* leaf inoculation method.

MATERIALS AND METHODS

The experiment was conducted in Fruit Pathology Laboratory of the Department of Fruit Science, Punjab Agricultural University, Ludhiana in 2022. A total of 31 hybrids were tested. Among the 31 hybrids, 18 were developed from sour orange (SO, female parent) × Rangpur lime (RL, pollen parent) cross and 13 from its reciprocal scheme, *i.e.* RL (female parent) × SO (pollen parent). The hybridity of these hybrids was verified previously through polymorphic SSR markers (Kaur *et al.*, 11). The SO × RL hybrids and RL × SO hybrids have been designated with acronyms SR and RS, respectively and the accompanying suffix indicates their identity of production. The plants of parents and tested hybrids were raised asexually through cuttings. For *Phytophthora* screening, fully expanded leaves were collected from these cutting-derived plants. The mycelial bits of 4.0 mm^2 were cut from the periphery of cultured virulent *P. nicotianae* isolate and were placed upside down on the top of each leaf pre-punctured by a sterilized needle.

The experiment was carried out in a completely randomized design (CRD) with three replications and each replication with three leaves. Uninoculated leaves were kept in control. The control leaves were mock-inoculated with a bit of sterile corn meal agar medium. The inoculated leaves were placed on a wet filter paper fixed in a petri dish. The petri dishes were covered with their lid and black carbon paper, followed by incubation at 25 ± 2°C for three days. After three days, the data were recorded on length (mm), breadth (mm) and area/ size (mm^2) of necrotic lesions. From the necrotic lesion size or area, the susceptibility/ relative tolerance of parents and hybrids was depicted using Yan *et al.* (19) scale. In this scale, the smaller lesion size corresponds to better tolerance against the pathogen.

The data were subjected to analysis of variance for testing the significance and differences between the means were analyzed with Tukey's HSD test at 5% level (p=0.05). The principal component analysis was also performed to determine the relative contribution of lesion length, lesion breadth and lesion area in total variation.

RESULTS AND DISCUSSION

In mock-inoculated leaves, no necrotic expansion was observed; hence, these were not considered for statistical analysis. *Phytophthora* induced variable length lesions of 5.9 to 45.0 mm and 2.8 to 46.9 mm in the inoculated leaves of parents and hybrids, respectively (Table 1). The highest average lesion lengths of 46.9 and 46.6 mm were recorded in the hybrids SR 32 and SR 13, respectively, indicating them to be the most sensitive to *Phytophthora* infection in lesion length assay. Rangpur lime, the susceptible parent and hybrid RS 9 shared statistical similarity to these hybrids. The hybrids SR 3 and RS 6 displayed the lowest necrotic lengths of 2.8 and 3.2 mm, respectively. The tolerant parent sour orange with 5.9 mm long lesion shared statistical parity with these hybrids. Lesion length is an important variable for determining the severity of the disease and depicting the resistance of germplasm especially under *in-vitro* assays. Assessment of pathogenicity of *Colletotrichum siamense* and *Pestalotiopsis jesteri* in rubber clones *via in-vitro* detached leaf assays was also adjudged through the lengths of circular necrotic lesions produced by the fungi (Aliya *et al.*, 3).

Like lesion length, the hybrids SR 3 and RS 6 also developed significantly lowest lesion width (3.2 and 3.4 mm). The tolerant parent, sour orange with lesion breadth of 5.5 mm, showed statistical parity with the two hybrids. The widest necrotic spot was witnessed in SR 7 (26.9 mm) (Table 1). The susceptible parent, Rangpur lime and the hybrids, *viz*. SR 13, SR 32 and RS 9 with necrotic lesion of 20.9 to 21.8 mm width showed statistical parity with SR 7 (Table 1). The lesion breadth is vital in calculating the area of the tissue affected by the pathogen. Cacciola *et al.* (4) computed the degree of pathogenicity of *Colletotrichum ocimi* on *Ocimum basilicum* leaves based on both length and breadth of pathogen-induced lesions in an *in-vitro* assay.

The affected area of the leaf is considered a more reliable criteria for judging genotypic response as it takes into account both lesion length and breadth or width. The necrotic lesion area of the inoculated samples was calculated by multiplying the lesion length and breadth dimensions (Table 1). The hybrid, SR 3 had the least necrotic area of 9.2 mm^2. The tolerant parent, sour orange and 12 other hybrids (SR 10, SR 19, SR 23, SR 6, RS 6, RS 22, RS 7, RS 18, RS 1, RS 1, RS 3, RS 31 and RS 27) with necrotic lesions of size 12.7 to 97.6 mm^2 showed statistical resemblance to it. The hybrids SR 13 recorded the largest necrotic area of 1011.8 mm^2. The susceptible parent, Rangpur lime and a few other hybrids, SR 32, SR 7 and RS 9, also developed necrotic lesions

Table 1. *In-vitro* disease reaction of hybrids of Rangpur lime and sour orange to *P. nicotianae* infection.

Parent/ hybrid	Necrotic lesion dimension[#]			Degree of resistance[##]
	Length (mm)	Breadth (mm)	Area/ size (mm^2)	
Rangpur lime	45.0^{ab}	21.0^{abc}	939.4^{a}	HS
Sour orange	5.9^{jkl}	5.5^{ghijk}	36.4^{fg}	R
Sour orange × Rangpur lime (SR) hybrids				
SR 3	2.8^{l}	3.2^{k}	9.2^{g}	HR
SR 10	3.7^{kl}	3.5^{ijk}	14.3^{fg}	HR
SR 19	3.7^{kl}	3.9^{ijk}	15.0^{fg}	HR
SR 23	6.7^{jkl}	5.1^{hijk}	36.6^{fg}	R
SR 6	9.7^{hijkl}	4.2^{ijk}	59.0^{fg}	MR
SR 17	8.6^{ijkl}	9.1^{efghijk}	113.6^{efg}	S
SR 27	13.7^{ghijkl}	12.8^{cdefghi}	216.8^{defg}	HS
SR 31	17.0^{fghijk}	3.5^{bcdefgh}	342.6^{cdef}	HS
SR 14	18.8^{efghij}	11.3^{efghijk}	264.9^{cdefg}	HS
SR 1	19.0^{defghij}	15.0^{bcdef}	336.3^{cdefg}	HS
SR 35	22.1^{cdefghi}	20.1^{abcd}	446.6^{bcde}	HS
SR 12	22.4^{cdefgh}	12.0^{defghij}	287.6^{cdefg}	HS
SR 25	22.9^{cdefgh}	9.8^{efghijk}	295.8^{cdefg}	HS
SR 24	29.2^{cdef}	17.4^{bcde}	541.3^{bcd}	HS
SR 7	31.2^{cde}	26.9^{a}	952.9^{a}	HS
SR 2	32.7^{bcd}	16.8^{bcde}	567.0^{bc}	HS
SR 13	46.6^{a}	21.8^{ab}	1011.8^{a}	HS
SR 32	46.9^{a}	20.9^{abc}	983.3^{a}	HS
Rangpur lime × Sour orange (RS) hybrids				
RS 6	3.2^{l}	3.4^{k}	12.7^{fg}	HR
RS 22	3.8^{kl}	4.6^{ijk}	17.9^{fg}	HR
RS 7	4.8^{kl}	4.7^{ijk}	23.8^{fg}	R
RS 18	5.0^{kl}	4.8^{ijk}	34.2^{fg}	R
RS 1	5.7^{jkl}	4.3^{ijk}	25.7^{fg}	R
RS 3	6.6^{jkl}	7.0^{fghijk}	56.3^{fg}	MR
RS 31	7.0^{jkl}	7.1^{fghijk}	51.3^{fg}	MR
RS 27	9.6^{hijkl}	9.3^{efghijk}	97.6^{fg}	MR
RS 5	11.1^{hijkl}	10.0^{efghijk}	133.2^{efg}	S
RS 29	15.4^{ghijkl}	13.8^{bcdefg}	261.6^{cdefg}	HS
RS 2	15.9^{fghijkl}	15.3^{bcdef}	276.2^{cdefg}	HS
RS 37	27.3^{cdefg}	14.9^{bcdef}	506.8^{bcd}	HS
RS 9	33.8^{abc}	21.1^{abc}	715.8^{ab}	HS
HSD (p = 0.05)	13.7	8.5	333.1	-

[#]In mock-inoculated leaves, no necrotic expansion was observed; hence, these were not considered for statistical analysis. [##]Disease scale [Yan *et al.* (19)] necrotic area of size <20 mm^2: Highly resistant (HR), 20.1-50 mm^2: Resistant (R), 50.1-100 mm^2 : Moderately resistant (MR), 100-200 mm^2: Susceptible (S), >200 mm^2 : Highly susceptible (HS).

of size statistically similar to SR 13 (Fig. 1A). To validate that variability of lesion size was induced by *P. nicotianae*, the infected leaf bit was cultured on to corn meal agar medium. The retrieval of the pathogen, *Phytophthora* in the culture medium validated that *Phytophthora* infection was the main cause of lesion size variability, thus verifying the leaf assay results (Fig. 2 A-C).

The principal component analysis (PCA) was performed to find out the relative contribution of the three variables to overall variation. The lesion length and lesion area constituted PC1 while lesion width formed PC2. The PC1 explained 94.5% variation indicating lesion length and lesion area are more important determinants of disease reaction (Fig. 3). Based on the area of necrotic lesions, a relative tolerance rating has been given by Yan *et al.* (19). On this scale, the hybrids could be classified into highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S) and highly susceptible (HS) class. From the 18 SO × RL hybrids, three hybrids were rated as HR (SR 3, SR 10 and SR 19), one as R (SR 23), one as MR (SR 6) and the remaining hybrids were rated as S or HS to *Phytophthora* (Fig. 1F). Among the RL × SO hybrids, two hybrids belonged to HR class (RS 6 and RS 22) (Fig. 1C), three as R (RS 7,

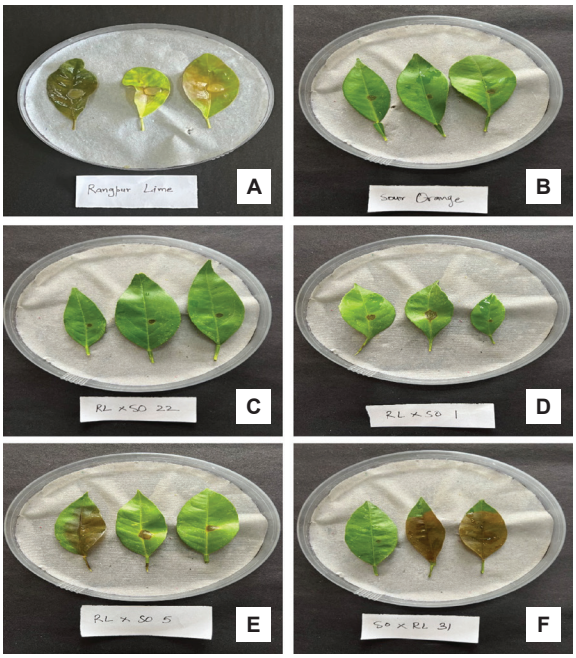

Fig. 1. Screening of parents and hybrids against *Phytophthora nicotianae* using detached leaf assay under *in-vitro* conditions, A: Rangpur lime (Highly susceptible); B: Sour orange (Resistant); C: RS 22 (Highly resistant); D: RS 1 (Resistant); E: RS 5 (Susceptible); F: SR 31 (Highly susceptible).

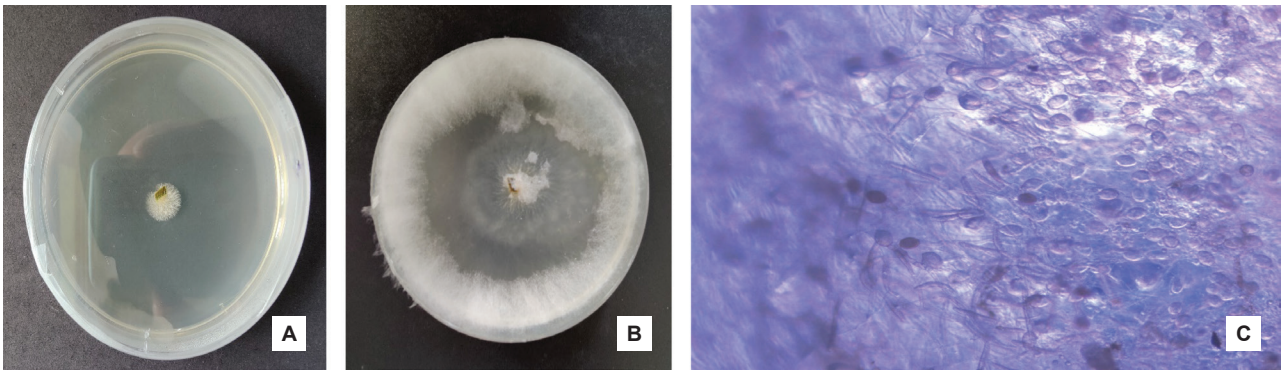

Fig. 2. Confirmation of *Phytophthora nicotianae* from the infected leaves through isolation. A: culturing of infected leaf bit on corn meal agar (CMA) medium; B: *P. nicotiana*e growth from infected leaf tissue C: Microscopic confirmation of the pathogen morphology.

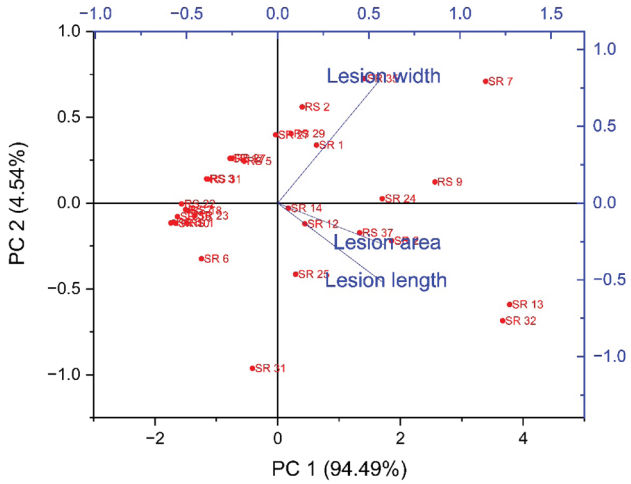

Fig. 3. PCA analysis of lesion length, lesion width and lesion area of the leaves following *Phytophthora* inoculation.

RS 18 and RS 1) (Fig. 1D), three as MR (RS 3, RS 31 and RS 27), one as S (RS 5; Fig. 1E) while remaining four were HS to *Phytophthora* infection. The parent sour orange displayed a resistant reaction (Fig. 1B). Thus, based on the Yan *et al.* (19) scale, five hybrids of this study showed *Phytophthora* resistance even better than that of the tolerant parent, sour orange. Earlier, Yan *et al.* (19) evaluated the *Phytophthora* tolerance of 22 citrus and eight *Poncirus* accessions based on the development of brown rot lesion areas in leaves post-pathogen inoculation. The lesion size response varied according to the genotype and a local mandarin (Guanggan) did not experience any disease necrosis on leaves. In their study, disease resistance predicted through leaf lesion attribute matched the resistance reaction obtained under artificial stem inoculations with the pathogen. Similarly, Herewini *et al.* (10) also found *in vitro* leaf screening useful in differentiating the kauri (*Agathis australis*) trees against *P. agathidicida* induced dieback. Thus, the estimation of lesion size based depiction of resistance can be considered a reliable estimate of relative resistance to *Phytophthora* in citrus.

From this study, SR 3, SR 10 and SR 19 displayed minimum necrotic length, breadth and area in SO × RL hybrids while RS 6 and RS 22 hybrids from the RL × SO group proved to be least affected by *P. nicotianae* under *in-vitro* leaf-inoculation based screening. The tolerance assessment of these selected hybrids through *in-vivo/* pot culture based screening methods will pave way for their commercial utilization.

AUTHORS' CONTRIBUTION

Conceptualization of research (KK, AA); Designing of the experiments (AA, HS); Contribution of the experimental material (KK); Execution of field/ lab experiments and data collection (HS); Analysis of data and interpretation (HS, KK); Preparation of the manuscript (HS, KK, AA).

DECLARATION

The authors declare that they do not have any conflict of interest.

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