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Original article

In vitro leaf inoculation as an early screening test for Citrus rootstock hybrids for *Phytophthora* root rot

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Summary

Introduction – **_Phytophthora nicotianae_ var. _parasitica_ is responsible for stem gummosis and root rot diseases in Citrus worldwide. Efficient utilization of the Citrus germplasm collection in India requires that these materials be evaluated for resistance to _Phytophthora_ species. The use of resistant rootstocks are the most economic and efficient method of _Phytophthora_ control.** *Materials and methods* – **In this study, In 2016, 10, 10 and 25 hybrids from the population of Rough lemon × X-639 citrandarin, Rough lemon × GouTou Cheng and Rough lemon × Swingle citrumelo respectively were identified on the basis of morphology and also confirmed by SSR markers.** *Results and discussion* – **Leaf baiting of hybrids with injury found to be better than without injury to leaf because comparison of lesion size was more accurate in this method. Use of Swingle citrumelo, Carrizo citrange and X-639 citrandarin as male parent resulted in higher resistance against the _Phytophthora_ in the progeny. The Rough lemon × Trifoliate orange rootstocks showed minimum lesion and sporangia in the leaf discs followed by Swingle citrumelo and X-639 citrandarin. All the rootstock hybrids showed higher PCV than the GCV and ECV which indicates greater role of phenotype in the expression of the character.** *Conclusion* – **Hybrids from the crosses Rough lemon × _Poncirus trifoliata_ and Rough lemon × Swingle citrumelo can be exploited to improve _Phytophthora_ resistance in Citrus.**

Keywords
rough lemon, Trifoliate orange, sporangia, SSR markers and zygotic seedlings

Abbreviations
RL (Rough lemon), X (X-639 citrandarin), GT (GouTou Cheng), CC (Carrizo citrange), PT (*Poncirus trifoliata*) and SC (Swingle citrumelo). XH-(Rough lemon × X-639 citrandarin), GTH-(Rough lemon × GouTou Cheng), CCH-(Rough lemon × Carrizo citrange), PTH1-(Rough lemon × *Poncirus trifoliata* 2013), PTH2-(Rough lemon × *Poncirus trifoliata* 2014), SCH1-(Rough lemon × Swingle citrumelo 2014) and SCH2-(Rough lemon × Swingle citrumelo 2015).

Significance of this study

What is already known on this subject?
- *Phytophthora* root rot is the main disease of Citrus and causes lesions on leaves and fruit, resulting in early defoliation. The density and distribution of the pathogens are influenced by high temperature combined with heavy soil and air humidity and therefore, vary across Citrus production areas.

What are the new findings?
- *Poncirus trifoliata* is indeed resistant to *P. nicotianae*. Surprisingly, it showed even resistance than did any of GouTou Cheng. In conclusion, hybrids from the crosses Rough lemon × *Poncirus trifoliata* and Rough lemon × Swingle citrumelo are a promising material that can be exploited to improve *Phytophthora* resistance in Citrus.

What is the expected impact on horticulture?
- Based on these findings, it is recommended to develop hybrids from cross between Rough lemon × *Poncirus trifoliata* have more resistance against *Phytophthora* as compared to other crosses.

Introduction

Citrus is one of the important fruit crops with great economic and health values around the world. Citrus fruits, cultivated under varied agro-ecological conditions, from arid and semiarid areas of southwest region to humid subtropical climate of north-eastern region of India, are presently plagued with various problems. Gummosis is one of the most important diseases affecting Citrus production in India and are responsible for 10 to 30 percent of losses in Citrus production worldwide (Alvarez *et al.*, 2011; Ahmed *et al.*, 2012; Meitz-Hopkins *et al.*, 2014; Graham and Feichtenberger, 2015; Panabieres *et al.*, 2016; Timmer *et al.*, 2000). Gummosis caused by fungus species, *viz.*, *Phytophthora palmivora*, *P. citrophthora* and *P. nicotianae* but, *P. nicotianae* was found to be the predominant species in India (Dhakad *et al.*, 2014; Das *et al.*, 2016). The density and distribution of the pathogens are influenced by temperature, soil type and relative humidity (Alvarez *et al.*, 2009; Timmer *et al.*, 2000), hence, it varies across the Citrus growing areas.

Selection or breeding for *Phytophthora* resistance is considered as the most efficient approach to manage the pathogen (Menge and Nemec, 1997). The use of resistant rootstocks is the most economic and efficient method of *Phytophthora* control (Feichtenberger, 2001; Alvarez *et al.*,

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2008; Hung *et al.*, 2015). Most of the Citrus breeding research has intended to select varieties that are resistant to diseases. A few genetic studies using a controlled progeny have been performed to understand the inheritance of citrus diseases such as *Phytophthora* root rot (Rauf *et al.*, 2013). Hutchison (1985) reported the complex and quantitative inheritance of *Phytophthora* resistance in Citrus. *Phytophthora* resistance has not been discovered in genus Citrus except the 'Guanggan' mandarin (Yan *et al.*, 2017). *Phytophthora* resistance has also been found in a Citrus relative *Poncirus trifoliata. Poncirus* has been utilized in breeding programme to develop new suitable Citrus rootstocks resistant to *Phytophthora*. *P. trifoliata* was used to map quantitative trait locus (QTLs) that confer resistance allowing the identification of three quantitative loci (Siviero *et al.*, 2006). It will take generations of conventional cross-hybridization to incorporate resistance without incorporating unwanted genes from *Poncirus* into Citrus (Menge and Nemec, 1997). Somatic hybrids developed by fusing Citrus cells with those of *P. trifoliata* (Guo and Deng, 2001; Mourao Filho *et al.*, 2008) are difficult to develop and regenerate. The process of developing a *Phytophthora* resistant rootstock can be substantially shortened using resistance genes from Citrus, if there is one in the genus (Siviero *et al.*, 2006).

The reason for lesser progress in breeding for *Phytophthora* resistance in Citrus may be due to poor selection techniques, lack of genetic variability in breeding programmes and probably an inadequate understanding of the genetic nature of resistance (Zhou *et al.*, 1997; Graham and Menge, 1999). Efficient utilization of the Citrus germplasm collection requires faster screening for resistance to *Phytophthora* species. In one of our studies (Singh *et al.*, 2017), leaf baiting was efficiently utilized for screening of Citrus genotypes against *Phytophthora*. The genotypes *Poncirus trifoliata*, Swingle citrumelo, X-639 citrandarin, and Gou Tou Cheng were found be tolerant to *Phytophthora.* Hence, it was hypothesised that the leaf bait technique can also be utilized for the fast screening of large progenies of new Citrus rootstock hybrids. In the present investigations, the Citrus rootstock hybrids developed after hybridizing 'Rough lemon' with *Poncirus trifoliata*, Swingle citrumelo, X-639 citrandarin, Carrizo citrange and GouTou Cheng were screened against *Phytophthora* using leaf bait technique.

Materials and methods

The Rough lemon (RL) plants on Rough lemon rootstock propagated with scion buds from a single elite Rough lemon tree planted in Rough lemon block, College Orchard, Department of Fruit Science, PAU Ludhiana, located at 29.3°N latitude and 76.5°E longitude, 270 m above mean sea level (a.m.s.l.) were used as the seed parent. The GouTou Cheng (GT) is a strain of Sour orange (*Citrus aurantium* L.), *Poncirus trifoliata* (TO), Carrizo citrange (CC: Washington sweet orange × Trifoliate orange), X-639 citrandarin (X; Cleopatra mandarin × Trifoliate orange), Swingle citrumelo (SC; Duncan grapefruit × trifoliate orange) plants planted at the nearby Fruit Research Farm of the Department of Fruit Science, PAU Ludhiana, were used as pollen parents. Anthers collected from the unopened flowers of the paternal parents just before anthesis, were desiccated with silica gel and pollen was stored at 4°C till use. Unopened fully developed flowers of RL were emasculated and pollinated with pollens of trifoliate orange hybrids during mid of March, 2014 and 2015. The RL fruits were harvested during the end of September, 2014 and 2015 at 180–190 DAP (Days after Pollination). The seedlings with multifoliate leaves at 4–5-leaf stage were transplanted to the black polythene bags (30 × 15 cm) filled with the same growing media and were kept under shade net house (50% shade).

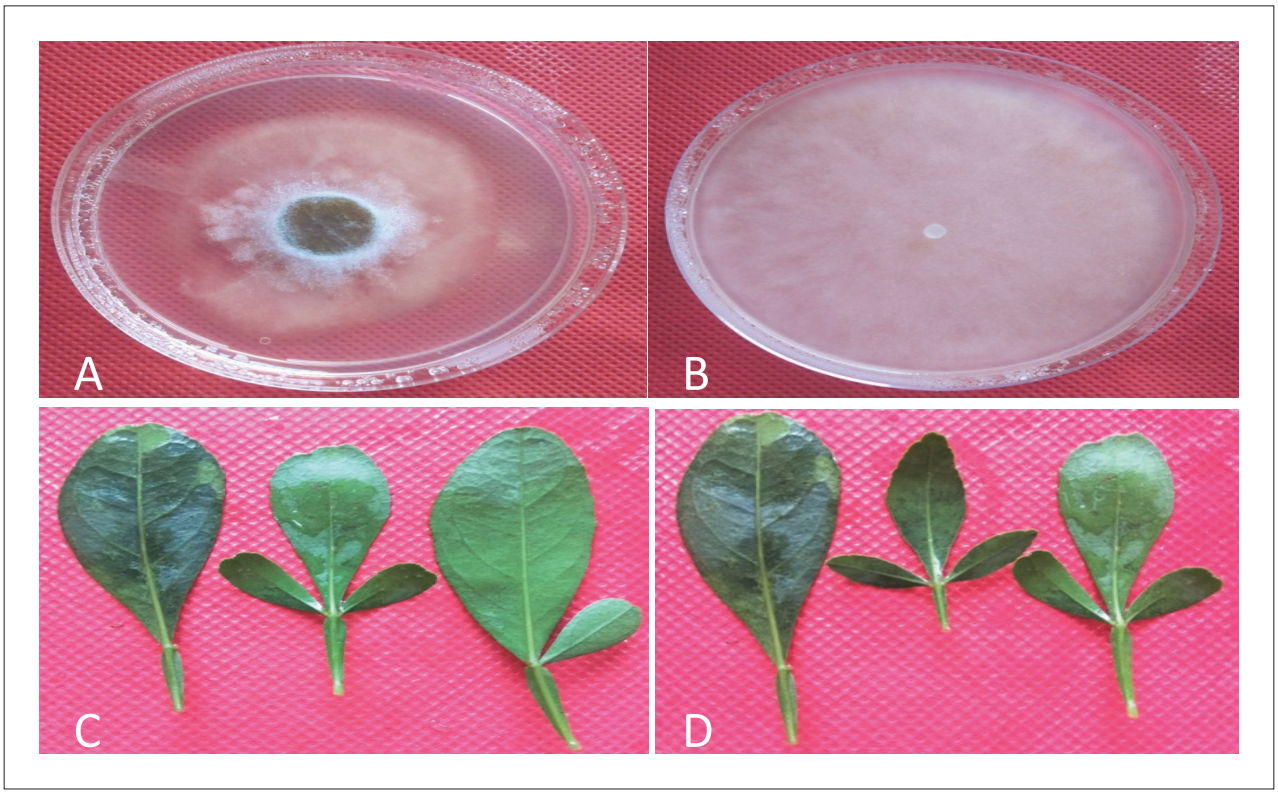

Figure 1. A) Culture of *Phytophthora nicotianae* var. *parasitica*; B) Mycelial growth and development of *Phytophthora* lesion (with and without injury) on C) RL × SC; D) RL × X.

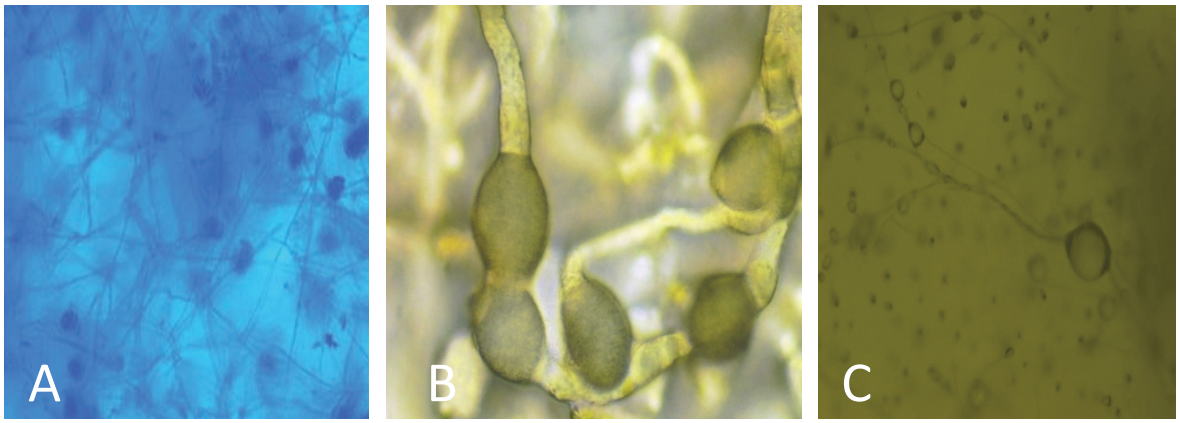

Figure 2. Morphological characteristics of *Phytophthora nicotianae* var. *parasitica*; A) Finely radiate cottony mycelium; B) Sporangium with prominent papilla; C) Sporangia on the edge of leaf disc bait after 96 h.

Isolation and multiplication of pathogen population

Pathogen was isolated (Figures 1 and 2) from root zone soil of Grapefruit plant infected with *P. nicotianae* and symptoms such as sap oozing from small cracks in the infected bark and yellow foliage and twigs die back, from College Orchard and was cultured on selective PARPH (Pimaricin-Ampicillin-Rifampicin-PCNB-Hymexazol) media (Naqvi, 1994) by using soil plating and leaf baiting method (Kannwischer and Mitchell, 1978; Ferguson and Jeffers, 1999). Corn meal agar (CMA, Himedia Laboratories Pvt. Ltd.) was used as basal medium which was prepared by adding 1 L of water in 17 grams of CMA. The medium was boiled and autoclaved at 121 ± 1°C for 20 minutes at 15 psi. Antibiotics solution was added to lukewarm corn meal agar media (CMA) pouring into the Petri plates. Multiplication of pathogen was done on sorghum seeds as described by Kaur *et al.* (2013). The autoclaved sorghum seeds were inoculated with 5 mycelial disc (5 mm) of pathogen from fresh culture. These flasks were incubated at 25 ± 1°C. After three days of incubation, mycelium growing on sorghum seed was dispersed by shaking. Spore suspension was made as described by Naqvi (2004) where forty seeds covered with mycelial after attaining the full growth of the pathogen suspended in Petri plates having 20 mL of sterilized deionized water. These plates were incubated at 25 ± 1°C. Water was replaced with fresh water for first two days. Abundant sporangia were formed in 3–4 days from mycelium.

Screening by leaf baiting technique (Harada and Kondo (2009))

Parents and their hybrid rootstocks were evaluated by using leaf baiting method for quick detection of resistance or susceptibility for pathogen. Spore suspension was made by taking forty seeds covered with mycelial growth of the pathogen in Petri plates with 20 mL of sterilized water. Approximately 100 mL of the suspension solution at a density of approximately 1×10^4 zoospores mL L^{-1} was added to each Petri plate. Twenty mL spore suspension was mixed with 80 mL of water in a beaker and fresh leaves were taken from 8 to 36 months old seedlings of each hybrid and their parents. Injury to the leaves was made by sterilized needle before baiting. Observations were recorded as lesion size and the number of sporangia on leaf disc after 48 hours up to 120 hours. The data recorded during 2016 were pooled and subjected to biometric analysis. Genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), environmental coefficient of variation (ECV), heritability (h2), genetic advance (GA) and correlation among lesion size at different hours were estimated with the help of SAS 4.5 software.

Results and discussion

The present investigation included different intra-specific crosses, *viz*., RL × TO, RL × CC, RL × X and RL × SC having bifoliate and trifoliate leaves and RL × GT having unifoliate leaves. Trifoliate leaf is used as a morphological marker to discriminate hybrids from a nucellar seedling. During 2014, maximum number of hybrids (25) having trifoliate leaves were identified from the cross (RL × SC), followed by RL × TO, RL × CC. In 2016, 10, 10 and 25 hybrids from the population of RL × X, RL × GT and RL × SC respectively were identified on the basis of morphology and also confirmed by SSR markers. Zhu *et al.* (2013) have also identified 43 and 14 zygotic seedlings, respectively from Shantou-Suanju × Flying Dragon and Shantou-Suanju × Xiaoganzhi, under *in vitro* conditions from mature seeds.

Response of explants (leaf with injury) to suspension of *P. nicotianae* inoculation

The observations recorded on lesion size on entire leaf with injury (RL × X) after 48 hours of incubation showed that XH-2 exhibited a minimum lesion size of 0.41 cm whereas XH-3 but XH-9 (1.48 cm) showed maximum lesion size (Supplementary Figure 3). The rootstock XH-4 showed minimum lesion size (1.20 cm) after 96 and (1.91 cm) 120 hours of incubation. Screening of F_1 population (RL × GT) against *P. nicotianae* var. *parasitica* by leaf bait technique has been presented in Supplementary Figure 4. Significant differences were recorded in lesion size on entire leaf with injury incubated with zoospore suspension of hybrids (RL × GT) were tested with respect to their parents injury. On the hybrid seedlings, the lesions developed earlier. Reduction in lesion size on entire leaf was observed in 7 hybrids out of 10 hybrids over their female parent, *i.e*. Rough lemon (Supplementary Figure 4).

In vitro screening of zygotic seedlings consisting of recombinants derived from a cross between RL and CC were evaluated in response to infection by *P. parasitica* (Table 1).

Table 1. Screening of citrus rootstock hybrids (RL × CC) against *Phytophthora nicotianae* var. *parasitica* by leaf bait technique.

Parents and hybrids	Lesion size on entire leaf bait without injury (cm)				Lesion size on entire leaf bait with injury (cm)				Number of sporangia on leaf disc			
	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
RL	0.92	1.05	2.54	3.14	1.12	1.22	2.07	3.35	271.0	202.7	110.3	62.3
CC	0.52	0.70	1.32	2.31	0.75	0.90	1.63	2.31	216.3	154.3	84.0	26.0
CCH-1	0.30	0.62	1.30	1.81	0.62	0.82	1.49	2.10	149.3	96.0	72.3	23.7
CCH-2	0.41	0.68	1.32	1.92	0.72	0.91	1.50	2.22	210.0	124.3	82.7	25.3
CCH-3	0.72	0.81	1.90	2.62	0.91	0.92	1.91	2.50	235.7	180.3	94.3	44.3
CCH-4	0.52	0.72	1.56	2.12	0.81	0.92	1.82	2.49	217.0	160.7	88.7	33.7
LSD (0.05)	Hybrids = 0.06 Hours = 0.05				Hybrids = 0.06 Hours = 0.05				Hybrids = 5.38 Hours = 4.48			

The maternal parent had the highest lesion length (0.92 cm) after 48 hours using entire leaf bait with injury. Hybrid number CCH-1 showed least lesion size (0.30 cm) after 48 hours of incubation of wounded leaf bait with zoospores. After 96 and 120 hours of incubation CCH-1 also showed minimum lesion size as 1.49 cm and 2.10 cm with injury, respectively. The hybrid rootstocks, *viz*., PTH1-5 and PTH1-8 showed maximum lesion size (above 2.0 cm) and SCH1-17 (1.47 cm). Zygotic seedlings developed from hybridization of RL × PT (2014, Supplementary Figure 5) and RL × SC (2015, Table 3) showed significant differences in lesion length on entire leaf with injury after 48, 72, 96 and 120 hours after inoculation with spore suspension. The RL × PT zygotic seedlings showed minimum lesion size and highest tolerance to *Phytophthora* because the *P. trifoliata* possessed genes responsible for tolerance to *Phytophthora* (Chen *et al.*, 2008).

Response of explants (leaf without injury) to suspension of *P. nicotianae* inoculation

Screening of Citrus rootstock hybrids (RL × PT) crossed in 2013 against *P. parasitica* was carried out using leaf bait technique. The F_1 population was initially developed for

Table 3. Screening of citrus rootstock hybrids (RL × SC) against *Phytophthora nicotianae* var. *parasitica* by leaf bait technique.

Parents and hybrids	Lesion size on entire leaf bait without injury (cm)				Lesion size on entire leaf bait with injury (cm)				Number of sporangia on leaf disc			
	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
RL	1.24	1.83	2.54	3.14	1.74	2.22	2.61	3.24	343.3	233.7	121.3	98.0
SC	0.73	0.92	1.64	2.31	0.92	1.33	1.70	2.42	287.0	151.7	58.3	34.7
SCH2-1	0.41	0.62	1.03	1.82	0.62	0.82	1.32	1.94	248.7	129.7	42.3	25.7
SCH2-2	0.00	0.00	0.51	0.91	0.31	0.62	0.83	1.33	198.0	90.3	26.3	17.0
SCH2-3	0.71	0.92	1.45	2.13	0.73	1.22	1.55	2.10	273.7	145.3	57.7	32.7
SCH2-4	0.31	0.51	0.82	1.64	0.51	0.72	1.13	1.75	237.3	125.7	35.0	19.7
SCH2-5	0.93	1.44	1.84	2.44	1.22	1.54	1.75	2.63	292.7	171.3	68.3	44.3
SCH2-6	1.12	1.55	2.09	2.75	1.26	1.74	2.02	2.84	294.7	172.7	89.3	57.7
SCH2-7	1.25	1.85	2.57	3.18	1.74	2.25	2.67	3.32	344.0	236.0	122.0	98.7
SCH2-8	0.51	0.72	1.22	1.94	0.62	0.91	1.44	2.12	256.0	137.0	53.7	28.3
SCH2-9	0.83	1.21	1.74	2.12	1.03	1.33	1.85	2.32	283.3	152.3	59.3	35.0
SCH2-10	0.00	0.31	0.52	1.22	0.41	0.62	0.92	1.43	208.7	121.3	26.7	17.0
SCH2-11	0.62	0.83	1.44	2.12	0.72	1.03	1.55	2.25	273.3	144.7	56.7	31.7
SCH2-12	0.42	0.62	1.13	1.83	0.62	0.91	1.32	1.93	255.3	133.0	48.3	27.3
SCH2-13	0.83	1.23	1.73	2.35	1.21	1.33	1.83	2.43	285.3	155.3	65.0	36.3
SCH2-14	0.31	0.52	0.92	1.74	0.52	0.81	1.24	1.84	247.3	128.3	39.0	22.7
SCH2-15	0.52	0.72	1.23	2.05	0.72	0.92	1.54	2.15	258.7	144.0	55.7	29.7
SCH2-16	1.25	1.64	2.10	2.85	1.45	1.86	2.21	3.04	296.0	185.7	92.3	82.0
SCH2-17	0.93	1.44	1.84	2.34	1.22	1.44	1.74	2.51	287.3	156.7	68.3	41.0
SCH2-18	1.31	1.87	2.55	3.18	1.73	2.27	2.66	3.26	344.3	334.3	123.0	97.7
SCH2-19	0.00	0.32	0.62	1.23	0.42	0.71	1.03	1.61	219.7	122.7	29.7	19.0
SCH2-20	0.22	0.41	0.81	1.63	0.51	0.72	1.09	1.75	234.0	123.3	33.0	19.7
SCH2-21	0.82	1.01	1.64	2.33	0.92	1.32	1.74	2.54	277.7	148.7	59.0	33.3
SCH2-22	0.94	1.54	1.93	2.64	1.24	1.74	1.99	2.84	293.7	172.7	82.0	46.0
SCH2-23	0.42	0.71	1.13	1.83	0.62	0.91	1.42	1.93	255.7	134.7	49.3	27.3
SCH2-24	0.73	0.93	1.55	2.14	0.82	1.22	1.64	2.34	276.7	146.7	58.3	33.0
SCH2-25	0.31	0.52	0.83	1.73	0.52	0.72	1.23	1.75	239.3	126.7	37.3	21.7
LSD (0.05)	Hybrids = 0.06 Hours = 0.02				Hybrids = 0.061 Hours = 0.024				Hybrids = 6.07 Hours = 2.34			

Table 2. Screening of citrus rootstock hybrids (RL × PT) against *Phytophthora nicotianae* var. *parasitica* by leaf bait technique.

Parents and hybrids	Lesion size on entire leaf bait without injury (cm)				Lesion size on entire leaf bait with injury (cm)				Number of sporangia on leaf disc			
	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
RL	1.00	1.32	2.03	2.42	1.22	1.32	2.84	3.14	276.7	226.0	192.3	101.7
PT	0.41	0.61	1.12	1.67	0.62	0.82	1.43	1.77	245.7	181.7	109.3	60.7
PTH1-1	0.30	0.50	0.71	1.31	0.50	0.61	1.01	1.51	205.7	143.3	77.7	26.3
PTH1-2	0.29	0.50	0.70	1.19	0.49	0.60	0.91	1.51	198.7	99.7	72.3	23.7
PTH1-3	0.60	0.81	1.21	1.72	0.71	0.90	1.51	1.90	266.0	201.3	145.7	74.0
PTH1-4	0.40	0.69	1.08	1.50	0.51	0.71	1.20	1.71	233.0	187.3	92.3	46.7
PTH1-5	1.04	1.34	2.04	2.42	1.22	1.33	2.85	3.15	274.0	226.3	191.0	101.0
PTH1-6	0.41	0.71	1.19	1.60	0.61	0.80	1.30	1.76	245.3	190.7	109.0	57.0
PTH1-7	0.20	0.41	0.70	1.01	0.40	0.60	0.90	1.21	178.3	90.7	54.3	17.0
PTH1-8	0.90	1.12	1.80	2.11	1.09	1.30	1.80	2.42	270.3	204.7	178.0	102.3
PTH1-9	0.61	0.90	1.28	1.81	0.80	0.91	1.70	1.90	266.7	203.3	166.3	86.7
PTH1-10	0.31	0.51	0.79	1.48	0.50	0.70	1.10	1.60	210.7	154.3	84.3	44.7
PTH1-11	0.50	0.71	1.21	1.61	0.70	0.89	1.41	1.81	262.67	200.67	134.33	68.33
LSD (0.05)	Hybrids = 0.05 Hours = 0.03				Hybrids = 0.06 Hours = 0.03				Hybrids = 6.42 Hours = 3.65			

early stage identification of zygotic seedlings through morphological (bifoliate and trifoliate marker) and SSR markers. Significant differences were recorded in lesion length of different hybrids and their parents (Table 2). Out of eleven, only five hybrids showed reduction in lesion size on entire leaf over the susceptible parent RL and also over the tolerant parent PT. The visual observation on entire leaf without injury showed that minimum lesion size (0.20 cm) was observed in hybrid number PTH1-7. Maximum lesion size was observed for PTH1-5 (1.04 cm) after 48 hours of incubation. After 72 hours of incubation, the minimum lesion size (0.41 cm) was for PTH1-7 while it was maximum for PTH1-5 (1.34 cm). After 96 and 120 hours of incubation, PTH1-7 showed minimum lesion size as 0.70 cm and 1.01 cm, respectively. Screening of F_1 hybrids of a population consisting of recombinants derived from a cross between RL × X was done using leaf bait technique. Rough lemon which has been used as a female parent during present studies, has great interest as a rootstock because it imparts resistance to blight, psorosis, high production and good fruit quality in kinnow mandarin, but its use is very limited due to its highly susceptibility to *P. nicotianae*. No lesion developed in XH-5 up to 48 hours of incubation. Maximum lesion size was observed for XH-9 (1.32 cm) and XH-3 (1.43 cm) after 48 hours of incubation. The hybrid rootstocks, *viz*., XH-3 and XH-9, showed maximum lesion size (> 2.80 cm). The rough lemon parent represented the extremes in lesion length on non-wounded leaf (2.82 cm) 120 hours after *P. nicotianae* incubation.

Screening focused on hybrids of RL × SC (2014), RL has great interest as rootstock because it confers resistance to psorosis, blight, high production and good quality fruit, but its use is very limited due to its high susceptibility to *Phytophthora* root rot. Therefore, Swingle citrumelo was used as male parent who imparts resistance to *Phytophthora*. *Phytophthora* inoculation was done using entire leaf method under *in vitro* conditions. Visual observation of lesion on leaf without injury, presented in Supplementary Figure 6 showed that no lesion was observed in hybrids (SCH1-7, SCH1-15, SCH1-21 and SCH1-25). After 96 and 120 hours of incubation, SCH1-7 showed minimum lesion size as 0.92 cm and 1.64 cm, respectively. The hybrid rootstocks, *viz*., SCH1-3, SCH1-8, SCH1-13 and SCH1-17 showed maximum lesion size (above 3.0 cm).

Response of explants (leaf disc) to suspension of *P. nicotianae* inoculation

The number of sporangia counted after 48 hours of incubation showed that all leaf baits of each hybrid (RL × X) rootstocks were attacked by large number of sporangia. Screening of hybrid seedlings against *Phytophthora* were also performed by leaf disc method in response to number of sporangia. The minimum number of sporangia after 48 hours was observed on XH-5 (165.67). The maximum number of sporangia after 48 hours was observed on leaf disc of XH-3 (285). The number of sporangia on each leaf disc of rootstocks decreased after 48 hours because sporangia germinated into mycelium on the edge of leaf disc (Singh *et al.*, 2017; Dhakad *et al.*, 2014). The reduction in the number of sporangia on leaf disc in XH-4, XH-5, XH-6 and XH-7 incubated on zoospore suspension indicates their tolerance against *P. parasitica*. Hybrid seedlings (RL × GT) were also screened by comparing number of sporangia on leaf disc. The minimum number of sporangia after 48 hours was observed on GTH-5 (229.3).

Hybrid seedlings (RL × CC) were screened against *Phytophthora* root rot by comparing number of sporangia on leaf disc with respect to their parents. After 48 hours of incubation number of sporangia counted from the leaf disc of hybrids and their parents showed that all leaf discs of each hybrid rootstock and parent were attacked by large number of sporangia. However, minimum number of sporangia after 120 hours was observed on CCH-1 (23.7). The maximum number of sporangia and was observed on leaf disc of CCH-3 (44.3). The Screening of Citrus rootstock hybrids (RL × PT, 2013) by counting number of sporangia on leaf discs showed that after 48 hours, the minimum number of sporangia were observed on PTH1-7 (178.3). According to these results, the reduction in the number of sporangia on leaf disc in PTH1-1, PTH1-2, PTH1-4, PTH1-7 and PTH1-10, incubated on zoospore suspension indicates their tolerance against *P. parasitica* as compared to other hybrids and their parents. The minimum number of sporangia after 48 hours was

observed on the leaf discs of PTH2-9 (205.7). Hybrid (RL × SC, 2014) rootstocks were evaluated against *Phytophthora* by comparing number of sporangia on leaf disc with zoospore suspension. The minimum number of sporangia after 48 hours was observed on the leaf discs of SCH1-7 (171.30). Citrus rootstock hybrids (RL × SC, 2015) were tested against *Phytophthora* root rot by comparing number of sporangia on leaf disc with zoospores. The minimum number of sporangia after 48 hours was observed on SCH2-2 (198.0). Comparison of resistance or susceptibility by counting sporangia does not seem familiar because all baits were attacked by abundant sporangia.

The mechanism of its resistance is most likely a local hypersensitivity reaction as cell death developed rapidly around the inoculation site on leaves as discussed by Yan *et al.* (2017). It is well-documented that such hypersensitive reaction can efficiently restrict the systemic spread of a virulent pathogen (Garcia-Brugger *et al.*, 2006; Nyadanu *et al.*, 2012). Similarly, Sharma (2000) and Harada and Kondo (2009) used leaf baiting for *Phytophthora* resistance in potato and adzuki beans, respectively. Dhakad *et al.* (2014) also observed maximum number of sporangia after 48 hours on the leaf discs of Rough lemon (531). During the present investigation comparison for *Phytophthora* resistance on the basis of number of sporangia on leaf disc was not found fruitful because after 48 hours, number of sporangia on leaf edge did not differ significantly. Dhakad *et al.* (2014) reported that Rough lemon and Cleopatra mandarin were found to be most susceptible, while Swingle citrumelo and X-639 citrandarin showed tolerant reaction against *Phytophthora*. This technique provides quick and easier results as compared to other screening methods. Leaf baiting of hybrids with injury found to be better than without injury to leaf because comparison of lesion size was more accurate in this method. The reduction in the number of sporangia on leaf disc and lesion length in the hybrid seedlings SCH2-2, SCH2-10, SCH2-19 and SCH2-20 indicates their tolerance against *P. nicotianae* var. *parasitica* as compared to other hybrids and their parents. Leaf baiting method of screening can be utilized for reaction against pathogen but, final consideration of hybrid reaction can be made by incubation of seedling by spore suspension.

Genetic parameters

The estimates of ECV (%), GCV (%), PCV (%) and GA for the *Phytophthora* reaction of five Citrus crosses is presented in Supplementary Figure 1. In RL × X the value of PCV was relatively higher (34.57%) than the GCV (34.19%) and ECV (4.87%), which indicates that the phenotype has played a greater role in the expression of the character (Supplementary Figure 1A). Similar, patterns were followed for all the crosses for ECV, GCV and PCV. The characters such as lesion size and number of sporangia, the difference between GCV and PCV was narrow. The extent of influence of these two determinants (environment and genotype) on the phenotype can be assessed from the magnitude of GCV and ECV (Hamidou *et al.*, 2018). The narrow difference is suggestive of the fact that phenotypic variation was determined by genotype with negligible influence of extraneous factors and selection for such characters will be rewarding (Khan *et al.*, 2006). The genotypes *P. trifoliata*, Swingle citrumelo, and Carrizo citrange can be utilized for the improvement of *Phytophthora* resistance in Citrus rootstocks under Indian conditions.

The high genetic advance coupled with high heritability (h^2 = 0.97%) of number of sporangia on leaf discs at 48 hours (GA = 62.69%) in RL × TO hybrids suggested appreciable level of improvement for *Phytophthora* resistance in the seedlings subjected to selection. Similar pattern was followed in other crosses like in RL × X (GA = 76.29%). The present findings are contrary to the low levels of heritability recorded earlier for resistance to *Phytophthora* (Siviero *et al.*, 2006; Chaudhary *et al.*, 2013). The contribution of individual characters towards divergence was assessed by ranking d1 (=Yi j-Yi k) values (Supplementary Figure 2A). In the present study, the maximum contribution towards the divergence was contributed by lesion size in the leaf discs without injury at 96 hours followed by leaf discs without injury at 48 hours. Number of sporangia after 48 hours at Cluster-3 followed by at Cluster-1 was the major contributor towards divergence in all the crosses. Hybridization between the rootstock seedlings selected from the diverse clusters is expected to express higher heterosis and produce desirable recombinants among the transgressive segregants (Dhillon *et al.*, 2009).

Conclusion

The trifoliate orange hybrids, namely Swingle citrumelo, Carrizo citrange, and X-639 citrandarin were more promising in developing hybrid Citrus rootstocks resistant against *Phytophthora*. Leaf baiting method of screening is a quick and easier technique to evaluate large number of seedlings against pathogens. This technique has the potential to initially screen the large number of hybrid seedlings in 48 hours but, final consideration of rootstock reaction can be made by incubation of seedling by spore suspension. Trifoliate orange showed higher resistant to *P. nicotianae* over GouTou Cheng.

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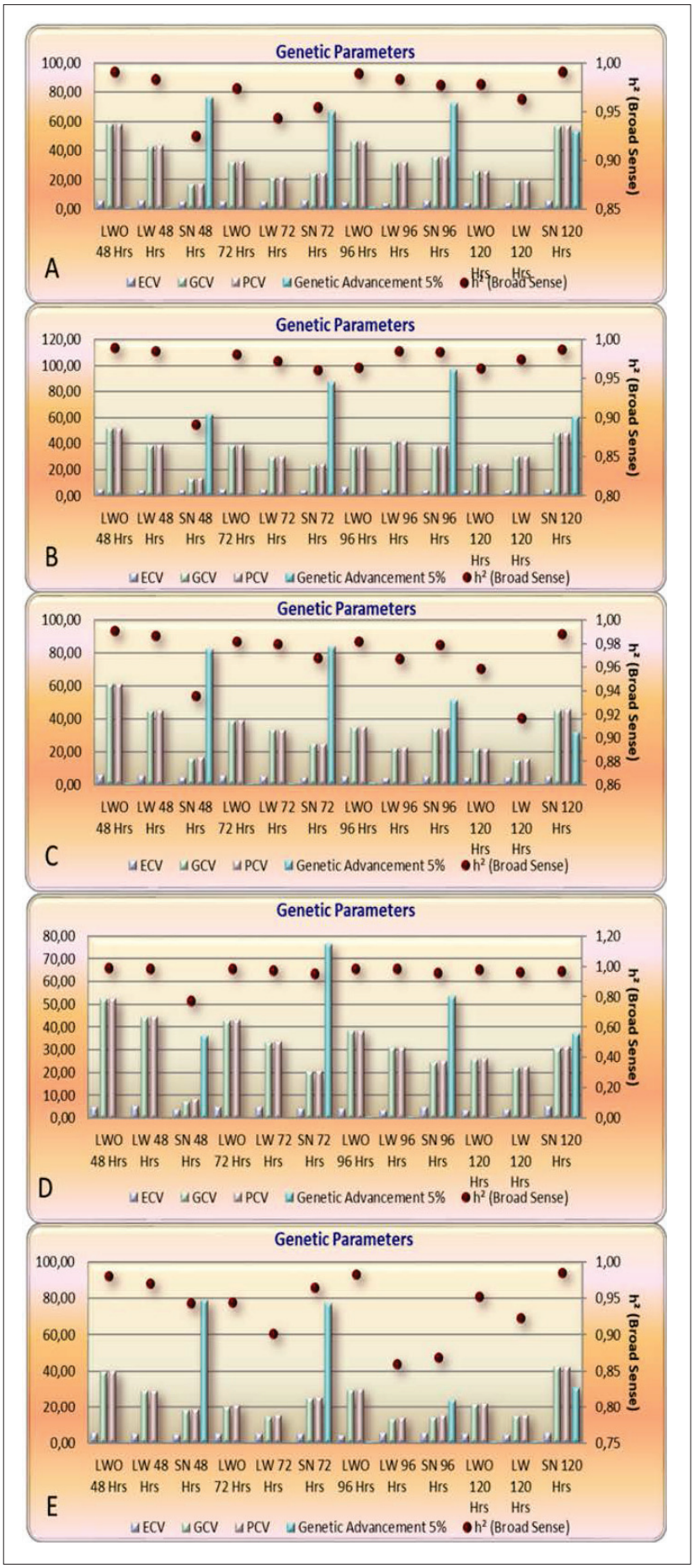
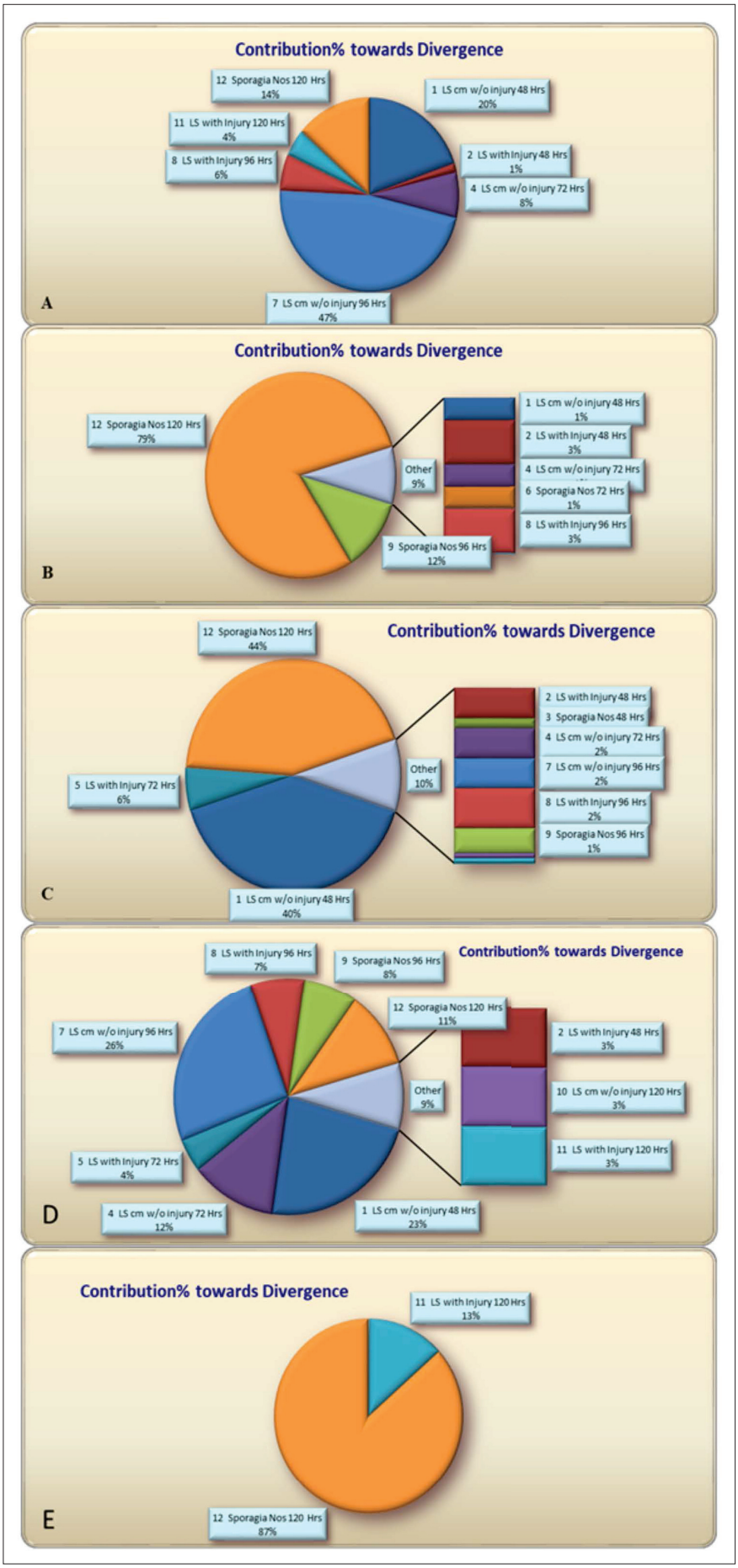
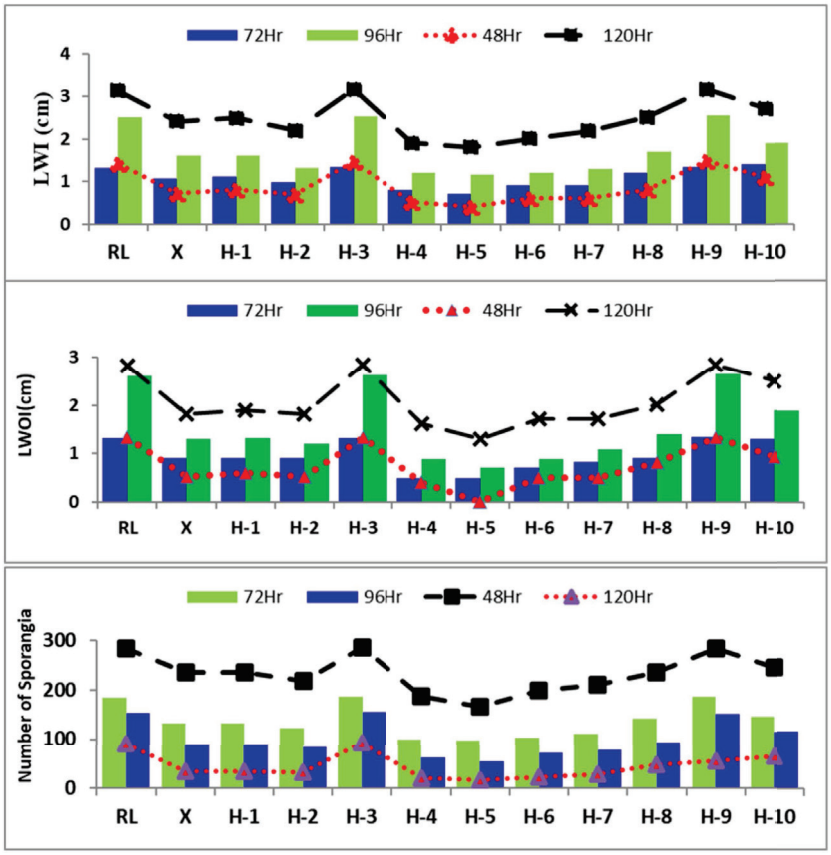


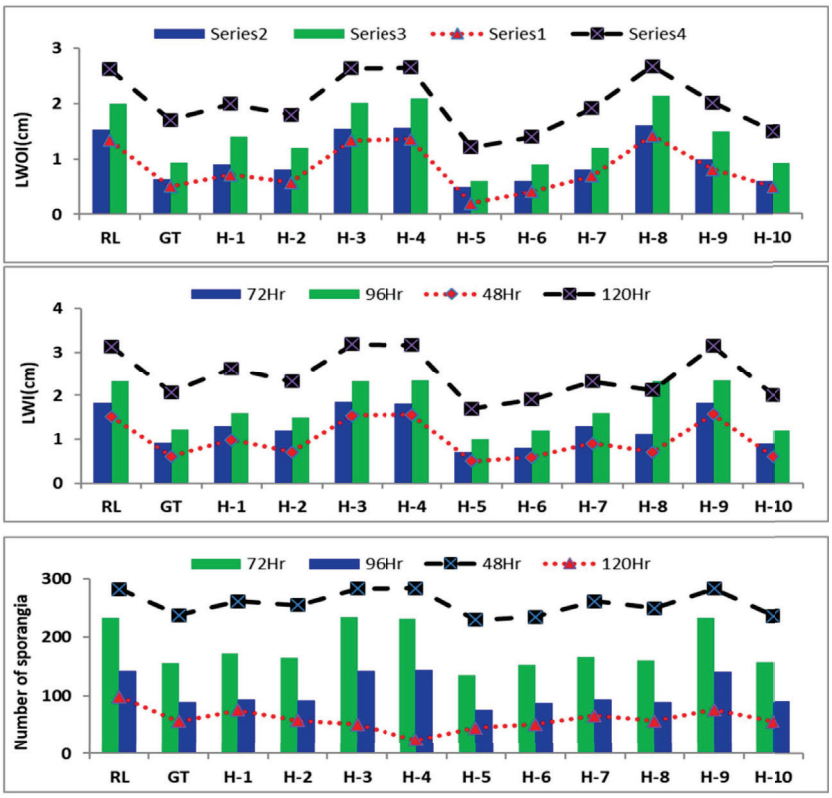
Supplemental Information – Figure S1. Genetic parameters A) XH; B) PTH1; C) SCH2; D) GTH; E) CCH.



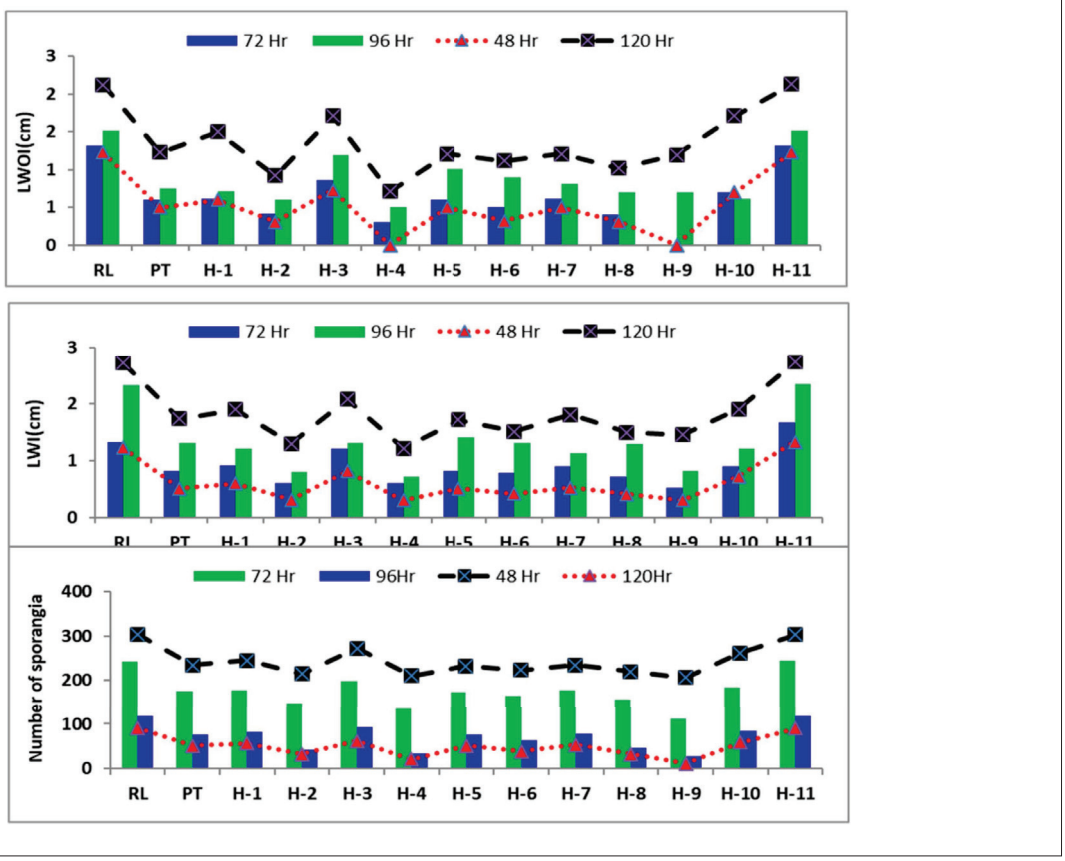
Supplemental Information – Figure S2. Contribution (%) towards divergence A) XH; B) PTH1; C) SCH2; D) GTH; E) CCH.



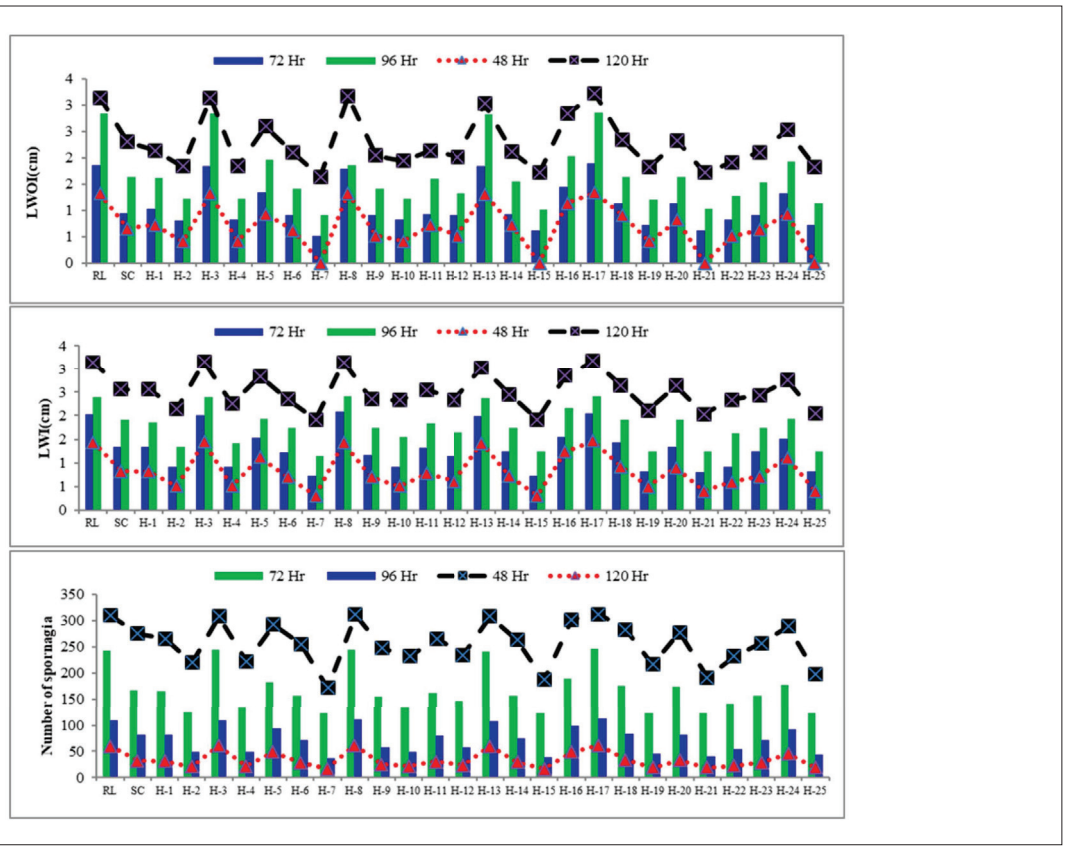
Supplemental Information – Figure S3. Screening of Citrus rootstock hybrids (XH) against *Phytophthora nicotianae* var. *parasitica* bij leaf bait technique.



Supplemental Information – Figure S4. Screening of Citrus rootstock hybrids (GTH) against *Phytophthora nicotianae* var. *parasitica* by leaf bait technique.



Supplemental Information – Figure S5. Screening of Citrus rootstock hybrids (PTH2) crossed in 2014 against *Phytophthora nicotianae* var. *parasitica* by leaf bait technique.



Supplemental Information – Figure S6. Screening of Citrus rootstock hybrids (SCH1) against *Phytophthora nicotianae* var. *parasitica* by leaf bait technique.