Scientia Horticulturae 172 (2014) 34–38

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Short communication

Monogenic recessive resistance to *Pepper leaf curl virus* in an interspecific cross of *Capsicum*

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ARTICLE INFO

Article history:
Received 4 December 2013
Received in revised form 14 March 2014
Accepted 25 March 2014

Keywords:
Artificial screening
Inheritance
Interspecific hybridization
Pepper leaf curl virus
Reproductive barrier

ABSTRACT

Pepper leaf curl disease is a serious threat to pepper production. Resistant sources based on field screening breakdown when virus pressure is severe. The lack of advanced screening techniques for *Pepper leaf curl virus* (PepLCV) limits the search for true sources of resistance. We standardized an artificial microcage inoculation technique and screened 22 pepper genotypes. Two earlier reported highly resistant sources, GKC-29 and BS-35, were confirmed, and Bhut Jolokia was identified as a new source of resistance. The inheritance study of resistance to PepLCV in a partially compatible inter-specific cross (PBC-535 × Bhut Jolokia) revealed monogenic recessive nature of PepLCV resistance. Bhut Jolokia may serve as a donor for the development of pepper cultivars with commercially acceptable fruit morphology and pungency.

1. Introduction

Pepper leaf curl disease, caused by whitefly-transmitted begomovirus (*Pepper leaf curl virus*; PepLCV) alone or coupled with thrips and mite infestation, constrains production of hot and sweet peppers worldwide, particularly in major pepper producing countries. India is the largest producer of dry chili fruit, accounting for more than 43% of the world's total dry chili production (FAOSTAT, 2011), however, 80–100% losses from leaf curling at the farmer's field have been reported (Prakash and Singh, 2006). Pepper leaf curl symptoms appear as curling of leaves, often accompanied by yellowing; the disease stimulates buds to produce clusters of small leaves, leading to stunted and bushy plants. Under extreme cases pollen development is hampered and flower buds abscise before attaining normal size, resulting in either no fruit set or setting of tiny fruits without commercial value. Virus strains from North India have been characterized and compared with other isolates from the Indian subcontinent (Rai et al., 2010; Senanayake et al., 2012) that revealed great virus diversity in this region.

The management of leaf curl diseases can be accomplished through judicious use of insecticides, border crops to control the vector, and virus resistant/tolerant cultivars. However, leaf curl resistant commercial cultivars are not available. We screened *Capsicum* germplasm against PepLCV in the field under natural epidemics and identified a number of field resistant lines (Kumar et al., 2011). Some of these lines were also screened against PepLCV through artificial screening, using a grafting technique to inoculate the virus (Kumar et al., 2006). However, the use of grafting for viral inoculation is time- and labor-intensive, with limited application to screen a larger number of genotypes and plants in segregating populations. These limitations necessitated the adoption of new screening techniques that can be used to screen larger populations. Given the fact that resistance in crop plants has mostly been identified in the weedy or wild relatives, an attempt was made to identify new sources of resistance in non-*Capsicum annuum* or interspecific landraces. We standardized an artificial screening method using the microcage technique to determine new sources of resistance, identify conventionally cross-compatible parents that produce fertile progeny, and understand the genetics of resistance against PepLCV, with the eventual goal of breeding of PepLCV resistant lines.

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http://dx.doi.org/10.1016/j.scienta.2014.03.039
0304-4238/

2. Materials and methods

2.1. Plant materials

From a list of *Capsicum* genotypes with known leaf curl disease reaction under open field conditions (Kumar et al., 2011), we selected four symptomless (SL), nine highly resistant (HR), one moderately resistant (MR), two susceptible (S), three each of moderately susceptible (MS) and highly susceptible (HS) genotypes for artificial screening using viruliferous whitefly inoculation and inheritance study if resistance in resistant sources was confirmed. Genotypes originated from six countries were comprised of landraces, released cultivars, exotic and indigenous collections and three naturally occurring inter-specific derivatives (BS-35, GKC-29 and Bhut Jolokia) (Table 1).

2.2. Populations for inheritance study

Immediately after confirmation of resistance sources through artificial inoculation during winter season (2008–09), attempts were initiated to hybridize BS-35, GKC-29 (HR) and Bhut Jolokia (R) genotypes with highly susceptible genotypes (Kashi Anmol, CCA-4261, PBC-535 etc.) on plant-to-plant basis. Since many crosses were unsuccessful, later efforts were focused on hybridizing Bhut Jolokia and BS-35 with PBC-535. The F_1 plants of two crosses (PBC-535 × BS-35 and PBC-535 × Bhut Jolokia) were raised in a nethouse (summer 2009), selfed to obtain F_2 seeds, and crossed with both parents to obtain backcross seeds (Table S1). Seedlings of parents, F_1s, F_2s and backcrosses (BC_1F_1) were raised during winter 2009–2010 inside an insect-proof nethouse for screening.

2.3. Inoculation methodology

Non-viruliferous *Bemisia tabaci* whiteflies were reared on plants of eggplant in a glasshouse (Fig. 1a). Virus (PepLCV-Varanasi) isolate was maintained on PepLCV susceptible sweet pepper (cv. California Wonder) plants kept in an insect-proof cage made of 50-mesh nylon net. Adult whiteflies collected from the eggplant plants were given an acquisition access period (AAP) of 24 h on the infected sweet pepper plants (Fig. 1b and c). Seeds of each genotype were sown in plastic trays filled with a mixture of soil, sand and farm yard manure, covered with microcages (Fig. 1d) and inoculated at the three-leaf stage, using 10–12 viruliferous whiteflies per seedling for an inoculation access period (IAP) of 24 h. Thereafter, to ensure uniform IAP to all plants, inoculated seedlings were sprayed with imidacloprid (0.3 ml/l) to kill the whiteflies. Seedlings were then transplanted in a vector-free greenhouse (Fig. 1e) and disease incidences were scored. For the inheritance study, five seedlings of parents and all the seedlings of F_1s, F_2s (56) and backcrosses with resistant (20) and susceptible parents (12) were grown in a nethouse along with infector rows (Kashi Anmol); about 1000 viruliferous whiteflies were released in the nethouse.

2.4. Measurement of disease severity

Disease reactions for each genotype were scored at 7, 14, 21, 28, 35 and 42 days post inoculation (dpi) on a symptom severity grade of 0 to 5 (modified from Kumar et al. (2006)) where 0 indicates no symptoms (symptomless, SL); (1) up to 5% curling and clearing of upper leaves (highly resistant, HR); (2) 6–25% curling, clearing of leaves and swelling of veins (resistant, R); (3) 26–50% curling, puckering and yellowing of leaves, and swelling of veins (moderately susceptible, MS); (4) 51–75% leaf curling, stunted plant growth and blistering of internodes (susceptible, S); (5) more than 75% curling and deformed small leaves, stunted plant growth with small flowers and no or small fruit set (highly susceptible, HS). After six weeks, a symptom severity grade was assigned to specific genotypes. The percentage leaf curling (PLC) data, scored weekly, was used to analyze disease progress in the tested genotypes.

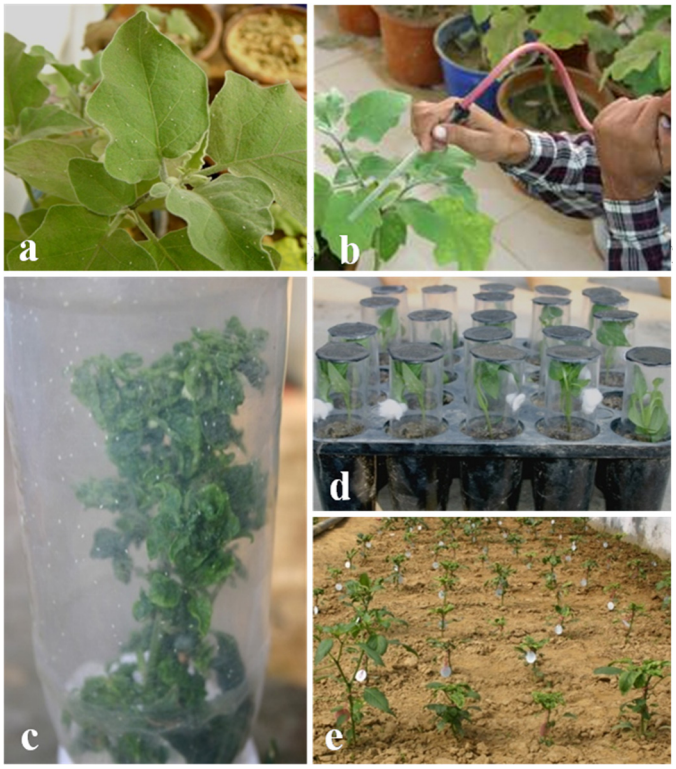

Fig. 1. Screening methodology for PepLCV resistance, (a) maintenance of whitefly population on eggplant, (b) collection of non-viruliferous adult whiteflies from leaf surface through aspiration, (c) releasing whiteflies for acquisition on severely infected sweet pepper plant for 24 h, (d) inoculation of 10–12 whiteflies on the test plantlets grown in plastic tray under microcages, and (e) transplanted plants after IAP of 24 h.

2.5. Diagnosis of virus

The presence or absence of viral particles in each genotype was also examined through polymerase chain reaction (PCR) with coat protein gene specific primers ChiLCV-CP (forward primer 5′-AGGGCTAAGGTCTAGATGTCCACACA-3′ and reverse primer 5′-TGTTCAATCACAACCTGAGGAAAGCG-3′), developed by Rai et al. (2010). Leaf samples were collected after 15 dpi and genomic DNA was isolated using DNAeasy plant mini kit following manufacturer's instructions (Qiagen Inc., USA). The PCR reaction mixture consisted of 50 ng of DNA, 1 U of Taq DNA polymerase (MBI Fermentas, USA), 1.5 μl of 10× PCR buffer, 0.5 μl of 10 mM dNTPs and 1 μl (10 pM) of forward and reverse primers. Routine PCR amplification (Rai et al., 2010) was followed and PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide (5.0 μg/100 ml) and visualized and documented using Alpha Imager 3400 gel documentation system (Alpha Innotech, USA).

2.6. Chi-square for goodness-of-fit

To understand the genetics of resistance in one successful cross, in all the segregating generations (F_2s, BC_1F_1s), individual plants were classified into resistant and susceptible categories (i.e. SL, HR and R plants were considered to be resistant while MS, S and HS plants were considered to be susceptible) as done by Munshi et al. (2008). A Chi-square (χ^2) test for goodness-of-fit was tested with the hypothesis of monogenic control of resistance to PepLCV from

Table 1
Reactions of *Capsicum* genotypes screened against *Pepper leaf curl virus*.

S. No.	Genotype (species)	Origin[a]	Field reaction[b]	% Leaf curling at 42 dpi	Reaction after inoculation	PCR test
1	BS-35 (*f* × *c*)	NER, India	SL	4.2 ± 0.37	HR	–
2	GKC-29 (*a* × *f*)	NER, India	SL	4.4 ± 0.24	HR	–
3	IC-383072 (*f*)	NER, India	SL	67.0 ± 1.00	S	+
4	Punjab Lal (*a*)	India	SL	83.0 ± 2.55	HS	+
5	Bhut Jolokia (*f* × *c*)	NER, India	HR	22.0 ± 1.22	R	+
6	Lankamura Collection (*f* × *b*)	NER, India	HR	68.0 ± 1.00	S	+
7	C00309 (*f*)	Taiwan	HR	66.0 ± 1.00	S	+
8	C00304 (*c*)	USA	HR	67.0 ± 1.58	S	+
9	NMCA-40008 (*f*)	NMSU, USA	HR	68.0 ± 1.22	S	+
10	NMCA-50003 (*a*)	NMSU, USA	HR	82.0 ± 3.74	HS	+
11	Taiwan-2 (*a*)	Taiwan	HR	86.0 ± 2.92	HS	+
12	DC-16 (*a*)	India	HR	81.0 ± 1.87	HS	+
13	Local Tripura (*a* × *f*)	NER, India	HR	83.0 ± 3.39	HS	+
14	Japani Longi (*a*)	India	MR	83.0 ± 2.55	HS	+
15	C05635 (*b*)	Brazil	MS	86.0 ± 1.87	HS	+
16	AMK-11 (*a*)	India	MS	85.0 ± 1.58	HS	+
17	PDG-50 (*a*)	India	MS	83.0 ± 2.55	HS	+
18	Pant C-1 (*a*)	India	S	84.0 ± 2.92	HS	+
19	Pusa Jwala (*a*)	India	S	85.0 ± 2.74	HS	+
20	CCA-4261 (*a*)	Taiwan	HS	86.0 ± 1.87	HS	+
21	PBC-535; Kashi Sinduri (*a*)	Indonesia	HS	85.0 ± 1.58	HS	+
22	KA-2; Kashi Anmol (*a*)	Sri Lanka	HS	86.0 ± 2.19	HS	+

a – *C. annuum*, *b* – *C. baccatum*, *c* – *C. chinense*, *f* – *C. frutescens*.
[a] NER – Northeast region, NMSU – New Mexico State University.
[b] Based on Kumar et al. (2011) and personal observations.

Bhut Jolokia. The genetic model was considered to be appropriate for a probability (*P*) value >0.05.

3. Results and discussion

3.1. Microcage inoculation technique and PepLCV resistant genotypes

In this study, a new microcage inoculation technique was standardized for artificial screening of PepLCV in chilli. After seven days of microcage inoculation, eight genotypes (BS-35, GKC-29, Bhut Jolokia, Lankamura Collection, C00309, C00304, NMCA-40008 and IC-383072) were symptomless, whereas disease symptoms started appearing on the younger leaves of the remaining 14 genotypes. The percent leaf curling (PLC) in all genotypes ranged from 4% (BS-35 and GKC-29) to 86% (KA-2) in six weeks. BS-35 and GKC-29 showed highly resistant reaction throughout the crop growth stage, with mild (4–5%) symptoms appearing at 28 dpi. Bhut Jolokia was reported to be symptomless under field conditions (Kumar et al., 2011); however, after artificial microcage inoculation, it showed resistance reaction. The remaining 19 genotypes under various categories of disease reaction in the field, turned out to be either highly susceptible or susceptible after 35–40 dpi (Table 1). Three highly susceptible genotypes under field conditions (Kumar et al., 2011; Table 1) remained highly susceptible at 35 dpi. In Bhut Jolokia, symptoms were first noticed at 15 dpi in one plant, while on the remaining four plants symptoms were noticed at 21 dpi. Two genotypes, IC-383072 and Punjab Lal, reported previously to be symptomless under field conditions (Kumar et al., 2011), turned out to be susceptible and highly susceptible, respectively. Among the susceptible category, five genotypes were susceptible and the remaining 14 genotypes were highly susceptible (Table 1). We thus further confirmed two highly resistant sources (BS-35 and GKC-29; Kumar et al., 2006) and identified Bhut Jolokia as a new source of resistance to PepLCV. Since seedlings were protected from virus before they were challenged with viruliferous whitefly, susceptible or highly susceptible reactions of 19 SL, HR, R, MR, S and HS genotypes based on a field screening (Table 1, Kumar et al., 2011) suggest that the inoculation technique we used was highly effective in transferring the virus particles. As compared to this microcage inoculation technique, graft inoculation technique is labor and time intensive, which limits its application in screening larger populations.

PCR results with geminivirus coat protein gene specific primers revealed that two highly resistant genotypes (BS-35 and GKC-29) did not show any amplification, re-confirming the absence of viral genomes in the symptomless plants as reported previously (Kumar et al., 2006). In contrast, all other genotypes including resistant Bhut Jolokia had amplification of a fragment of ~650 bp (Table 1), confirming the presence of the viral genome. The symptom development in highly susceptible genotypes began just after one week and rapidly progressed in six weeks, while in Bhut Jolokia there was a delay in the start (15 dpi) of symptom development and disease progress was slow. In two highly resistant landraces, BS-35 and GKC-29, disease advance was delayed (starting at 28 dpi) and reached only up to 4–5% in both genotypes after six weeks (Fig. 2), although in both landraces viral DNA was not detected through PCR. In principle, PCR is a powerful technique to detect DNA, but sometimes it fails to detect viral DNA reliably either due to presence of inhibitors in the plant extract (Accotto and Noris, 2007) or due to restriction in viral growth leading to availability of much less quantity of viral DNA for primer binding, as we used total plant DNA as template for PCR reaction. The resistant genotype, Bhut Jolokia, showed more sporadic disease symptoms than the susceptible genotypes with less than 25% leaf curling after 6 weeks dpi (Fig. 2). This type of resistance may be associated with the presence of mechanisms that inhibit either virus replication or movement, or both (Verlaan et al., 2013). All three highly resistant or resistant genotypes (GKC-29, BS-35, and Bhut Jolokia) are naturally occurring interspecific derivative landraces commonly grown in Northeast India and are well known for possessing high pungency (Bosland and Baral, 2007; Rai et al., 2013). GKC-29 also has been found resistant to anthracnose caused by *Colletotrichum capsici* (*Ccf*-Varanasi and *Ccc2*-Raichur) (Garg et al., 2013). Punjab Lal has been found to be resistant to *Pepper leaf curl virus*-Thailand strain (PepLCV-TH) at AVRDC – The World Vegetable Center and used in a crossing program to develop leaf curl resistant lines. PBC-535, a line resistant to bacterial wilt, was used as a resistant root

Table 2
Estimates of χ^2 and their probability based on a monogenic recessive control of PepLCV resistance in the cross PBC-535 × Bhut Jolokia.

Population	Segregation (number of plants)					Pooled segregation		Best fit ratio (R:S)	χ^2	P (5%)
	HR	R	MS	S	HS	R	S			
Bhut Jolokia (Pr)	4	6	0	0	0	10	0	–	–	–
PBC-535 (Ps)	0	0	1	3	6	0	10	–	–	–
F_1 (Ps/Pr)	0	0	0	1	5	0	6	–	–	–
F_2	3	9	18	22	4	12	44	3:1	0.38	0.65
BC_1F_1 (F_1/Pr)	4	7	1	6	2	11	9	1:1	0.20	0.81
BC_1F_1 (F_1/Ps)	0	0	1	10	1	0	12	0:All	0.0	1.00

Pr, resistant parent; Ps, susceptible parent.

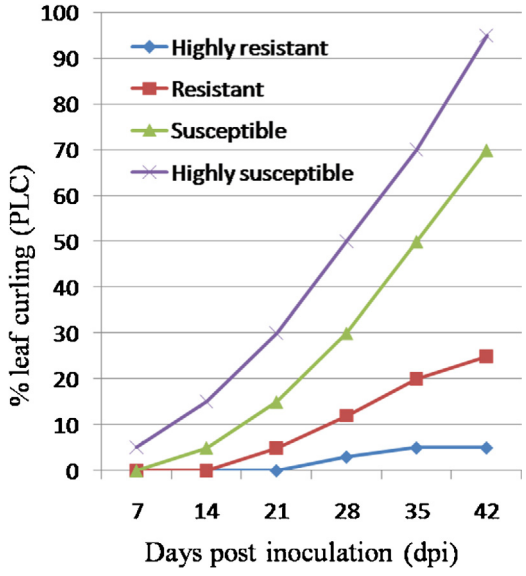

Fig. 2. Disease progress curve of PepLCV in highly resistant (BS-35, GKC-29), resistant (PBC-535), susceptible and highly susceptible groups.

stock. One of non pungent variants of PBC-535 was purified, evaluated and recommended for release in India as a paprika variety for oleoresin extraction.

3.2. Reproductive barriers between resistant and susceptible genotypes

Highly resistant (BS-35 and GKC-29) and resistant (Bhut Jolokia) inter-specific derivatives were crossed as staminate and pistillate parents with several susceptible genotypes. However, only PBC-535, a highly susceptible *C. annuum* genotype, was able to cross with limited success as a pistillate parent with Bhut Jolokia (5 F_1 plants) and BS-35 (2 F_1 plants) (Table S1). Despite more than 500 hybridization attempts, GKC-29 failed to cross with PBC-535 and no F_1 seed was obtained (Table S1). This suggests presence of pre-zygotic barriers between GKC-29 and PBC-535. Only two crosses (PBC-535 × BS-35 and PBC-535 × Bhut Jolokia) were successful, but in both the crosses, F_1 plants had very poor pollen fertility compared with their respective parents (Table S1). Only two of eight F_1 seeds of PBC-535 × BS-35 germinated and developed into mature F_1 plants. These two F_1 plants produced fruit with a total of 116 F_2 seeds, but all F_2 seeds failed to germinate (Table S1), suggesting the existence of hybrid breakdown (post-zygotic barrier) between BS-35 and PBC-535. Bhut Jolokia, was also crossed with PBC-535 with great difficulty, and eventually out of 12 F_1 seeds obtained from four crossed fruits, only five germinated and developed into F_1 plants. These five F_1 plants were successfully used to obtain 56 F_2, 20 BC_1F_1 with Bhut Jolokia and 12 BC_1F_1 with PBC-535 which were used to study genetics of resistance in Bhut Jolokia (Table 2).

Poor crossing success and the expressions of variable degrees of sterility in crosses and subsequent generations (Table S1) clearly revealed the expression of reproductive isolation mechanisms between *C. annuum* (PBC-535) and all three naturally occurring interspecific derivatives (GKC-29, BS-35, and Bhut Jolokia). These interspecific derivatives are believed to have originated from sympatric domesticated species in Northeast India (Rai et al., 2013). The existence of a set of pre- and post-zygotic barriers in *Capsicum* species is well-known (Hogenboom, 1973). Absence of pollen grain germination and the delay or inhibitions of pollen tube growth are the major pre-zygotic barriers, while embryonic death due to endosperm degeneration and total or partial sterility of hybrid plants are major post-zygotic barriers. All the individual F_2 seeds of PBC-535 × BS-35 were smaller than those of both parents and failed to germinate. For full embryo development, the endosperm has to reach a critical size as already suggested by Cooper and Brink (1942). This may be a reason for the failure of the F_2 seeds to germinate.

Likely, the interspecific *C. annuum* × *C. frutescens* hybrid derived GKC-29 was reproductively isolated from *C. annuum* cultivar (PBC-535) by pre-zygotic barriers, while the more distant *C. frutescens* × *C. chinense* (BS-35) showed evidences of having post-zygotic barriers with PBC-535. Comprehensive studies on the reproductive isolating mechanisms between *C. annuum* and inter-specific landraces are needed to understand speciation of the genus *Capsicum*, including naturally occurring allotetraploid *Capsicum* species (Jha et al., 2012) in the northeast Himalayan region.

3.3. Genetics of resistance

The F_1 plants of two successful crosses, PBC-535 (highly susceptible) × BS-35 (highly resistant) and PBC-535 × Bhut Jolokia (resistant) were found to be susceptible and highly susceptible (Table 2), respectively, indicating the recessive nature of resistance in both the resistant sources. Inheritance of resistance was analyzed in only one cross (PBC-535 × Bhut Jolokia) as the F_2 and backcrosse generations were successfully obtained and individual plants in both segregating and non-segregating generations were screened against PepLCV (PepLCV-Varanasi isolate; Rai, 2010). The 56 F_2 progenies segregated into 12 resistant and 44 susceptible plants, and the backcross to the resistant parent segregated into 11 resistant: 9 susceptible plants (Table 2). Backcross to the susceptible parent (PBC-535) progenies did not segregate and all 12 plants were susceptible (Table 2). Based on monogenic recessive control of the resistance trait, the expected ratio of the F_2 and testcross would be 3:1 (susceptible: resistant) and 1:1 (susceptible: resistant), respectively. The results of the Chi-square analysis indicated a good fit to both of these ratios (Table 2). In Bhut Jolokia, then, resistance is suggested to be under control of a single, recessive gene. We suggest further inheritance and marker analyses using larger populations before a gene symbol is proposed for the expression of leaf curl disease resistance in Bhut Jolokia in accordance with gene nomenclature for *Capsicum* (Wang and Bosland, 2006).

Information on the genetics of pepper leaf curl disease caused by PepLCV in the *Capsicum* germplasm is lacking. Although field resistant lines and inheritance of resistance under field conditions has been reported (Bal et al., 1995), to the best of our knowledge this is the first report of monogenic recessive resistance to PepLCV in a resistant source identified through challenged inoculation. In contrast, tremendous progresses have been made in crops like tomato, where at least five non allelic resistant genes to *Tomato yellow leaf curl virus* (TYLCV) have been identified and mapped on tomato genome (Kadirvel et al., 2013). As Bhut Jolokia has been found to be partially cross-compatible with *C. annuum*, we plan to initiate backcrossing program to transfer resistance into *C. annuum* backgrounds.

Acknowledgments

This work was supported through In-house grant to Indian Institute of Vegetable Research, Varanasi by Indian Council of Agricultural Research, New Delhi. The authors would like to thank Maureen Mecozzi, Head, Communication and Information, AVRDC – The World Vegetable Center for language editing of the manuscript. Authors thank to the anonymous reviewers for their constructive comments to improve the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2014.03.039.

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