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Selection of resistant source to early blight disease in tomato among the *Solanum* species

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Abstract

Resistance to early blight (EB) disease of tomato caused by *Alternaria solani* was assessed by examining various parameters of the disease progress. For this study twenty three diverse tomato genotypes were screened under replicated trials for over three years (2007-2009) using artificial inoculation under controlled conditions as well as under natural epidemics at Indian Institute of Vegetable Research, Varanasi, UP, India. Tested genotypes showed significant difference in their response to *A. solani* and disease severity. Area under disease progress curve (AUPDC) was positively correlated with percent disease index (PDI) and negatively with resistance. Of the 23 genotypes, only two *i.e.* EC-520061 (*Solanum habrochaites*) and H-88-78-1 (*S. lycopersicum*) were highly resistant (PDI < 5.0; AUDPC < 200 and r value ≥ 0.12) for EB disease under field and glasshouse environments. Characterization using molecular markers also indicated their resistance. It was concluded that there are significant differences between resistant and susceptible tomato lines against EB disease and some of the lines should be considered resistant rather than tolerant. Hence, the choice of resistant lines can be utilized in future breeding programmes for development of early blight resistant/tolerant cultivars of tomato.

Key words: Early blight, *Alternaria solani*, *Solanum habrochaites*, resistant, AUDPC

Introduction

Tomato (*Solanum lycopersicum* L. syn. *Lycopersicon esculentum* Mill.) is one of the most important vegetables grown worldwide. Besides several pests and diseases, the early blight (EB) caused by *Alternaria solani* (Ellis & Martin) Sorauer, is one of the most damaging foliar diseases capable to cause serious yield losses worldwide (Sherf and MacNab, 1986; Pandey *et al.*, 2003; Peralta *et al.*, 2005) and is highly correlated with the disease intensity (Dater and Mayee, 1985).

The control measures include crop rotation with non-hosts for several years, routine applications of chemical fungicides, and the use of disease-free germplasm (Madden *et al.*, 1978; Sherf and MacNab, 1986). Fungicide treatment is generally the most effective control measure but may not be effective under certain weather conditions favorable for epidemics (Herriot *et al.*, 1986). Conditions that favour early blight include heavy rainfall, high humidity, and relatively high temperatures (Chaerani and Voorrips, 2006). Due to health and environment concern, growers are looking for safer alternative approaches. Resistant cultivars are potentially the most economical and effective way to combat the disease as they can keep away and/or extend the fungicide spray intervals (Madden *et al.*, 1978; Shtienberg *et al.*, 1995; Keinath *et al.*, 1996). Cultivars highly resistant to early blight are not known in cultivated tomato (Chaerani *et al.*, 2007); however, some disease tolerant cultivars/lines are available (Nash and Gardner, 1988; Gardner and Shoemaker, 1999; Upadhyay *et al.*, 2009). Moreover, much vegetable cultivar evaluation focus on yield and fruit quality attributes with less emphasis on disease tolerance.

The molecular markers identified and characterized for early blight resistance in wild tomato species using interval mapping and selective genotyping approaches is immense beneficial for breeding programmes (Foolad *et al.*, 2000). One approach is the identification of candidate resistance genes using polymerase chain reaction (PCR) technology and sequence information from known disease resistance genes (R genes). Hence, it is imperative to screen tomato cultivars/lines against early blight disease caused by *A. solani* to identify sources of resistance having desirable qualitative and quantitative characteristics which could be utilized in traditional breeding approaches. Keeping this in view the objectives of the study were to screen tomato lines possessing good horticultural traits under natural epidemics and artificially inoculated controlled conditions, and to assess the disease progress with time and its relationship with the age of tomato plant.

Materials and methods

Twenty three phenotypically diverse tomato genotypes including 20 *S. lycopersicum* and three wild species (*S. pimpinellifolium S. glandulossum* and *S. habrochaites*) were obtained from the gene bank of Indian Institute of Vegetable Research, Varanasi, India (Table 1). All the 23 genotypes were screened under field and artificial (screen house) condition for three consecutive years (2007-09) at the research farm of IIVR, Varanasi. For field evaluation, twenty one days old seedlings were transplanted in sick-plots (2.5 × 2.0 m). Twenty plants per plot of each tomato line were maintained with three replications in a randomized complete block design (RCBD) at row to row and plant to plant spacing of 60 and 45 cm, respectively. Experiments were repeated twice during the study in field. No fungicide or other chemical was used throughout the study period. All standard practices were adopted to raise a good tomato crop. In field tests, EB severity was assessed in terms of percent defoliation and the average portion of necrotic leaf area on the plant (Horsfall and Barrat, 1945).

Table 1. Description of tomato genotypes tested against early blight disease caused by *Alternaria solani*

Genotypes	Source	Species	Growth habit	Selection based on the character
Agata-30	NBPGR, New Delhi, India	*S. lycopersicum*	Determinate	Fruit size and number of fruits
Agata-32	NBPGR, New Delhi, India	*S. lycopersicum*	Determinate	Fruit size and number of fruits
CO-3	TNAU, Coimbator, India	*S. lycopersicum*	Determinate	Light green large fruit & good yield
DT-10	IARI, New Delhi, India	*S. lycopersicum*	Determinate	Fruit oval, normal standing plants, more number of fruits
DVRT-2	IIVR, Varanasi, UP., India	*S. lycopersicum*	Determinate	Large fruit size and round shape
EC-520061	NBPGR, New Delhi, India	*S. habrochaites*	Indeterminate	Wild, long, aromatic, high TSS and more number of fruits.
EC-521080	NBPGR, New Delhi, India	*S. pimpinellifolium*	Indeterminate	Wild, long, high TSS and more number of fruits
FEB -2	NBPGR, New Delhi, India	*S. lycopersicum*	Determinate	Round fruit
FLA-7171	John Scott, Univ. Florida	*S. lycopersicum*	Determinate	Medium fruit size and round in fruit shape
FLA-7421	John Scott, Univ. Florida	*S. lycopersicum*	Determinate	Large round fruit, attractive
H-24	HAU, Hissar, India	*S. lycopersicum*	Determinate	Normal in fruit size and good yield
H-86	IIVR, Varansi, UP, India	*S. lycopersicum*	Determinate	Large fruit and good yield
H-88-78-1	IIVR, Varansi, UP, India	*S. lycopersicum*	Indeterminate	Cultivated , high TSS and good yield
H-88-78-2	IIVR, Varansi, UP, India	*S. lycopersicum*	Indeterminate	Cultivated, round and hard fruits.
H-88-87	IIVR, Varansi, UP, India	*S. lycopersicum*	Indeterminate	cultivated line, good yeild
KT-15	IARI, New Delhi, India	*S. lycopersicum*	Determinate	More number of fruits and medium size fruits
PKM-1	TNAU, Coimbator, India	*S. lycopersicum*	Determinate	Bunchy type plants and attractive dark green colour
Punjab Chhuhara	PAU, Ludhiana, India	*S. lycopersicum*	Determinate	Long fruit, good yield and attaractive
Roma	IARI, New Delhi, India	*S. lycopersicum*	Determinate	Medium fruit size and oval in shape, with light green colour
SEL-7	HAU, Hissar, India	*S. lycopersicum*	Determinate	Large fruit size and round in shape
Shalimar-2	SKAT, Srinagar, J&K, India	*S. lycopersicum*	Determinate	Medium and oval fruit
Sikkim Local	Sikkim, India	*S. lycopersicum*	Indeterminate	Cultivated, long plant height and good yeild
WIR-3928	NBPGR, New Delhi, India	*S. glandulossum*	Indeterminate	Wild, high TSS

For screen house studies, a total of 230 earthen pots were filled with sandy loam soil (soil, sand and farm yard manure in 2:1:1 ratio) and kept in randomised block design, maintaining 10 plants for each tomato line. The screen house was maintained at 22±2^{0}C and more than 95% relative humidity (RH). Experimental designs and replications were same for other screen house experiments which were also repeated twice during the study.

The pure culture of *A. solani* was raised on potato dextrose broth (PDB) in 500 mL conical flasks. Three hundred mL of PDB was autoclaved in a vertical type autoclave at 15 psi for 20 minutes. After sterilization the flasks were observed for a period of 48 h to ensure no contamination. Inoculation with pure culture of tested fungus was carried out under aseptic condition followed by incubation for two weeks at 26±1ºC. Both fungal mat and broth were thoroughly mixed with the help of electric mixer and grinder. An inoculum concentration of 125 cfu/mL was maintained and uniformly sprayed on four week old tomato plants (Pandey *et al.*, 2003) maintained in 6 inches diameter plastic pots containing approximately 1 kg sandy loam soil. The plants were kept in screen house and regularly fertilized with Hoagland solution. All the observations were recorded at 24 h interval based on the disease severity on zero to five (0-5) point scales: 0-Free from infection; 1-One or two necrotic spots on a few lower leaves of plant; 2-Few isolated spots on leaves, covering nearly 5%–10% of the surface area of the plant; 3-Many spots coalesced on the leaves, covering 25% of the surface area of the plant; 4-Irregular, blighted leaves and sunken lesions with prominent concentric rings on the stem, petiole, and fruit, covering 40–50% of the surface area; 5-Whole plant blighted, leaves and fruits starting to fall. The disease severity in terms of PDI, AUDPC and r were recorded as per Pandey *et al.* (2003).

$$\text{PDI}= \frac{\text{Sum of all ratings}}{\text{Total number of observations} \times \text{maximum rating grade}} \times 100$$

The mean value of PDI was calculated and host plant reaction was categorised on the following basis: 0-5%-highly resistant (HR); 5.1-12%-resistant (R); 12.1-25%-moderately resistant (MR); 25.1-50%-moderately susceptible (MS); 50.1-75%-susceptible (S) and >75%-highly susceptible (HS).

The area under disease progress curve (AUDPC) was calculated using the following formula:

$$AUDPC = \sum_{i=1}^{n-1}[(\frac{X_{i+1} + X_i}{2}) * (t_2 - t_1)$$

Where, X_i is the disease index expressed as a proportion at the i^{th} observation; t_i is the time (days after planting) at the observations and n is the total number of observations and rate of infection (r) was calculated as:

$$r = \frac{1}{t_2 - t_1} \log_e \frac{n_2(1-n_1)}{n_1(1-n_2)}$$

Where, t_1 is the time (days) during the first observation; t_2 is the time during the second observation; t_2 - t_1 is the time interval between two observations and subsequently; n_1 is the PDI at time t_1; and n_2 is the PDI at time t_2.

Data analysis: Data on disease severity and development were pooled and subjected to ANOVA using SPSS Ver. 12.0. Differences between treatments were determined by Duncan's Multiple Range Test at 5% significance level.

PCR reaction for screening through molecular markers: Total genomic DNA was extracted using modified CTAB protocol (Doyle and Doyle, 1990) from young leaves of six tomato genotypes that showed highly resistant, resistant and highly susceptible disease reactions. The master mix consisted of 1.0 µL dNTPs (containing 10 mM each dNTPs), 2.0 µL $MgCl_2$ (25.0 mM

$MgCl_2$), 0.75 µL Taq polymerase, 2.5 µL 10X reaction buffer, 1.0 µL of forward and reverse primer and 1.0 µL of genomic DNA. The PCR programme followed one cycle of pre denaturation at 94 ^{0}C for 4 min, 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 50-55 ^{0}C for 1 min, extension at 72 ^{0}C for 1 min, and one final extension cycle at 72^{0}C for 10 min. The amplification products were separated on 2.0% agarose gels (Bredemeijer *et al.*, 2002). The gels were analyzed using a gel documentation system- Alpha Imager (Alpha Innotech, USA). A large set of SSR primers were surveyed to find the polymorphic primers between the appropriate resistant and susceptible tomato lines.

Results

Tomato lines were screened for early blight disease under natural epidemic and screen houses by artificial inoculation under controlled conditions and the disease severity was compared in both the environments. Of 23 screened tomato lines (Table 1), four lines with indeterminate growth habit [EC-520061 (*S. habrochaites*), EC-521080 (*S. pimpinellifolium*), H-88-78-1 (*S. lycopersicum*) and WIR-3928 (*S. glandulossum*)] were recorded resistant against early blight under both the environments on the basis of PDI, AUDPC and r value. The lines EC-520061 and H-88-78-1 were highly resistant under both environments while response of EC-521080 and WIR-3928 differed with the environments (Fig. 1-3). Among these four lines, three (EC-520061, EC-521080 and WIR-3928) belong to wild species whereas H-88-78-1 is a cultivated line but derivative of *S. habrochaites f. glaboratum*, which seems to be the first report that shows high resistance against early blight disease. The mean value of PDI, r and AUPDC of all tested materials were higher under controlled conditions compared to open field condition (Fig.1a, b and c). The mean PDI of highly resistant lines (EC-520061 and H-88-78-1) was <5.0% (*i.e.* 0.0 and 4.75%). For resistant lines (EC-521080 and WIR-3928) the value of PDI was between 5.1 to 12% (Fig. 1a). It is noticeable that all the four resistant lines had indeterminate growth, and remained disease free for a longer period in the field without fruit or with few fruits. Being cultivated, H-88-78-1 line has some desirable characters that can be utilised for further improvement in the breeding programs. Likewise the value of infection rate was also recorded minimum in case of highly resistant lines followed by resistant lines (0.00 to 0.11). The infection rate was positive under both (field and screen house) conditions as the disease severity clearly increased between the first and last observation. The mean infection rate of all tested materials was higher under controlled conditions compared to field condition (Fig.1b). Under natural infection in field the AUDPC of the highly resistant variety EC-520061 was 0.0 and it was 23.1 when inoculated with *A. solani* under artificially controlled conditions. In case of other resistant lines it ranged from 137.5 to 206.4 under natural epidemics and 158.9 to 303.8 under artificially inoculated controlled conditions. Tomato lines that showed susceptible and highly susceptible reaction had extremely high AUDPCs, under both environments (Fig.1c). Most of the tested lines with good yield characteristics were either susceptible or highly susceptible under both the conditions. Susceptible lines were easily recognised by symptoms like infected stem, leaves, calyx, petiole etc. and the infection gradually increased leading to progressive increase in PDI with each observation. On the basis of disease parameters, line CO-3 had the highest PDI and AUDPC followed by Punjab Chhuhara. The PDI values were highest for highly susceptible lines under both natural (70%) and artificially inoculated controlled (79%) environments. Susceptible lines were easily infected and had extremely high AUDPC (1661 to 3240) under naturally infected field conditions. Negative correlation between the AUDPC and degree of resistance against pathogen was noted whereas a positive correlation was recorded between AUDPC and PDI which seems to increase with susceptibility. Most of the susceptible lines were determinate except H-88-78-2 and H-88-87 which were moderately susceptible, however, few of them (Punjab Chuhara, DVRT-2 and Roma) had qualitative and quantitative desirable characters and are already under cultivation.

The disease progress was also studied for both susceptible and resistant lines for two different time intervals (7 to 28 days and 45 to 90 days, respectively). For this study three lines from each group: EC-520060, H-88-78-1 and WIR 3928 of resistant group and CO-3, Punjab Chhuhara and Sel-7 of susceptible group were examined. Both groups of inoculated plants had distinctly different curves (Fig. 2a and b) at both time intervals. Initially, the curve seemed to increase rapidly followed by a gradual increase in case of susceptible lines. However, in case of resistant lines it was very slow and did not have any peak. It shows that high degree of resistance does not allow the causal organism to infect the plant rapidly. It was observed that the disease progress under both environment were almost similar at the initial stage but differed at the later stage as disease progressed in due course of time. The total area under disease increased gradually from resistant to highly resistant and susceptible to highly susceptible lines. Line Sel-7 reaction to *A. solani* was different from other lines during initial days of observation (Fig. 2a). The disease severity of susceptible lines increased with time, up to 90 days (Fig. 2b).

In another set of experiments, the effect of tomato plants age on PDI and AUPDC was also studied (Fig. 3a and b). It is notable that young plants (45 days) were less susceptible to *A. solani* infection compared to older (60 days) plants. The mean PDI and AUDPC values for most tested genotypes were much higher in older plants with highest value in case of CO-3 (up to 68.77 and 1247.25, respectively) compared to young plants (34.86 and 470.03, respectively). The apparent infection rate between two subsequent observations was more informative than the total infection rate in terms of disease spread. It was low in resistant varieties compared to susceptible ones. A positive correlation was observed between the disease incidence and age of plant under both conditions.

The SSR primers were used for polymorphic survey between resistant and susceptible lines with monomorphic or multiple bands products. These SSR markers were discarded and polymorphic primers repeated twice. Under present study, 15 SSR primers (SSR 104, SSR 108, SSR 112, SSR 124, SSR 156, SSR 210, SSR 218, SSR 226, SSR 241, SSR 304, SSR 308, SSR 310, SSR 316, SSR 350 and SSR 356) gave polymorphism against EC-520061 and were monomorphic against other tomato lines *viz.*, Punjab Chhuhara, H-88-78-1, Sel-7, CO-3 and WIR-3928 revealed that EC-520061 is most polymorphic source to use for development of resistant lines/varieties against early blight in tomato (Fig. 4).

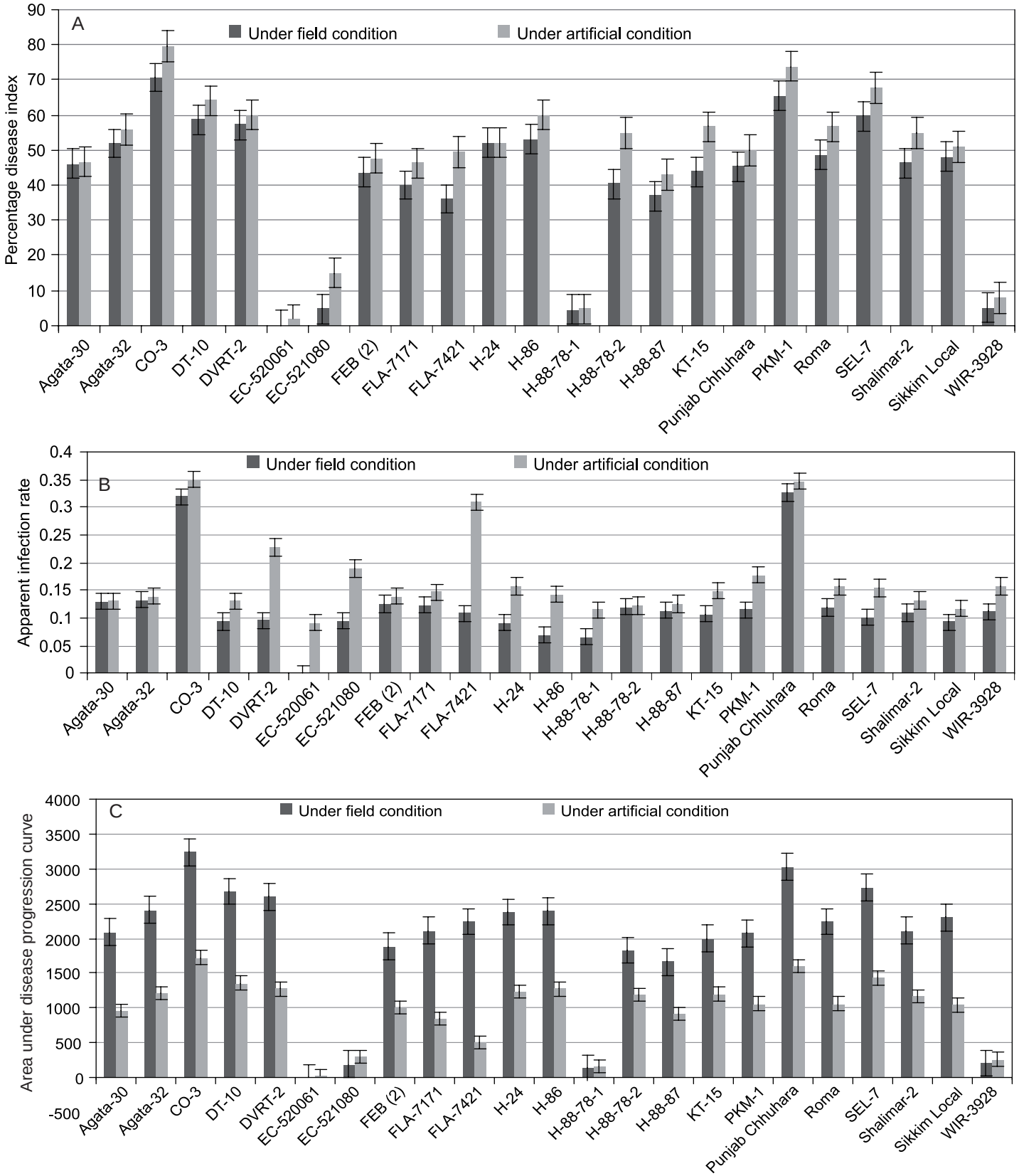

Fig. 1. Effect of early blight disease of tomato caused by *A. solani* on different genotypes under naturally infested field and artificially inoculated conditions (A) percentage disease index, (B) apparent infection rate and (C) area under disease progress curve. Vertical lines represent ± standard error.

Discussion

It is reported that the indeterminate growth habit of tomato crops has negative relation to biotic and abiotic stresses. It may be due to their continuous growth and physiological or biochemical changes in plants life cycle. In our study, the highly resistant (EC-520061 and H-88-78-1) and resistant (EC-521080 and WIR-3928) lines also had indeterminate growth habit. These results confirm the disease relation with plant growth habit in most accessions as reported by Pandey *et al.* (2003). Lines, H-88-87, H-88-78-2

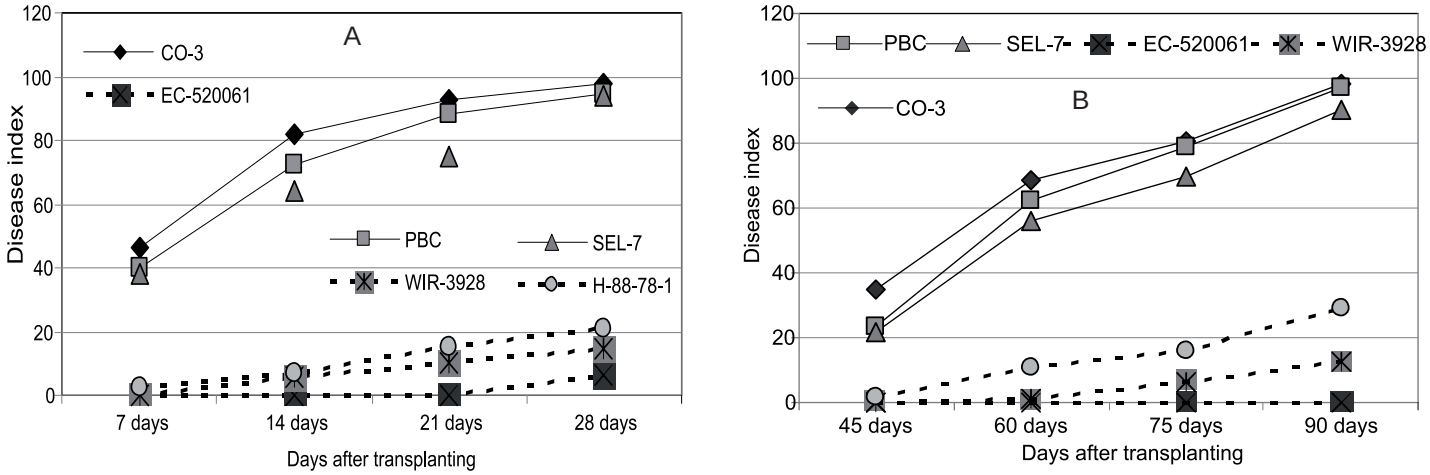

Fig. 2. Disease progress curve for early blight disease of tomato caused by *A. solani* on selected susceptible and resistant lines (A) Under artificially inoculated conditions up to 28 days and (B) Under naturally infected field condition up to 90 days.

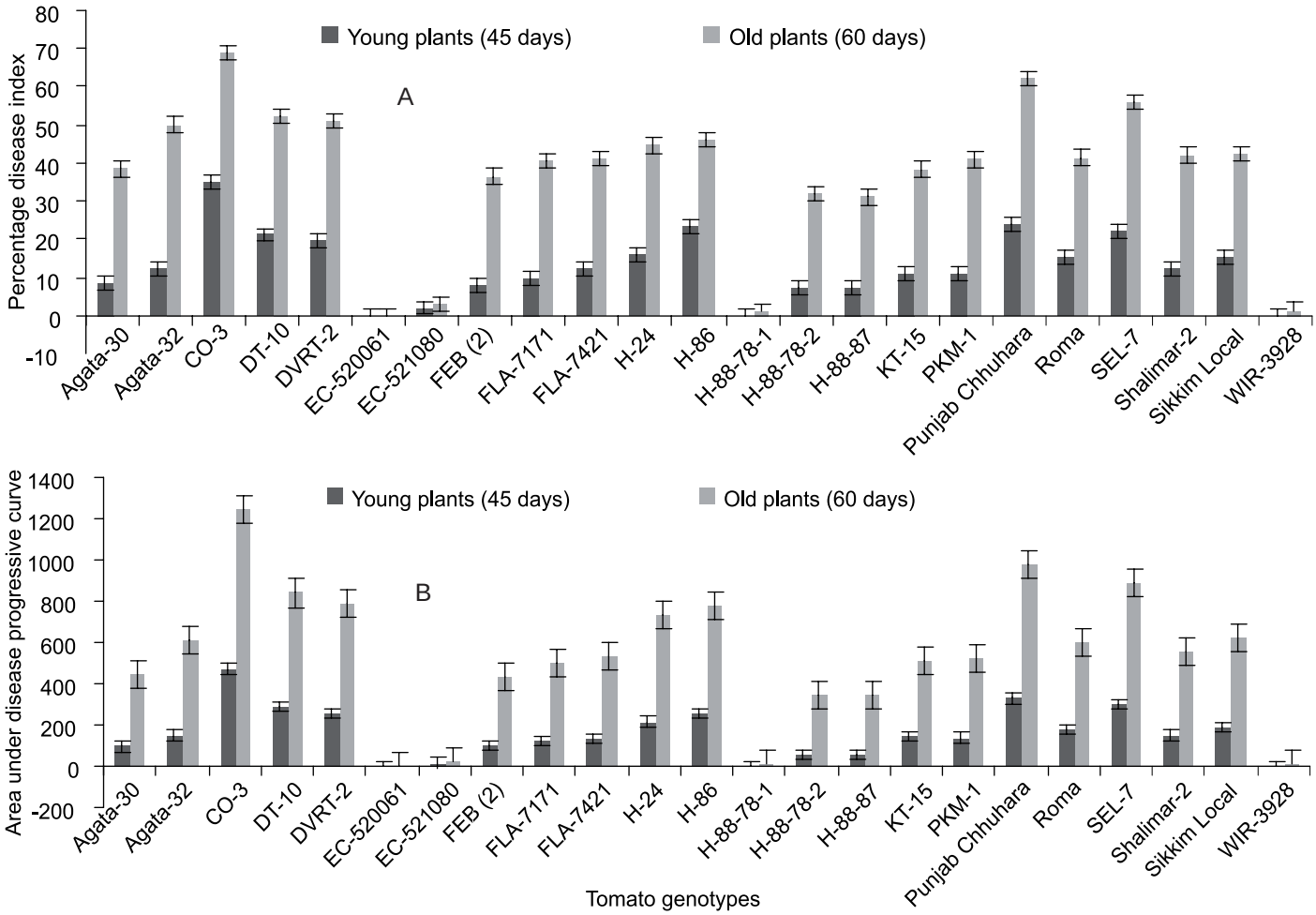

Fig. 3. Relationships between age of tomato genotypes with (A) Percentage disease index of early blight disease of tomato caused by *A. solani* (B) Area under disease progress curve of early blight disease of tomato caused by *A. solani*. Vertical lines represent ± standard error.

and Sikkim Local have indeterminate growth habit but showed moderately susceptible disease reaction, due to their slow growth habit compared to other indeterminate accessions at each stage. Whereas, Agata-30, FEB-2, FLA-7171, FLA-7421, H-24, PKM-1 and Roma have determinate growth habit and able to hold-up during an epidemic period without being much affected by the disease and also possess acceptable qualitative and quantitative characters (Foolad *et al.*, 2000).

Manual screening under field condition is a primary procedure for screening but controlled (artificial) condition screening is considered as better alternative for getting reliable information for EB disease (Pandey *et al.*, 2003). Various methods have been used for evaluation of plants for disease resistance by many workers (Kalloo and Banerjee, 1993; Pandey *et al.*, 2003; Upadhyay *et al.*, 2009). Natural epidemics of early blight are strongly influenced by environmental conditions, even though severe disease appears every year in northern India (Pandey *et al.*,

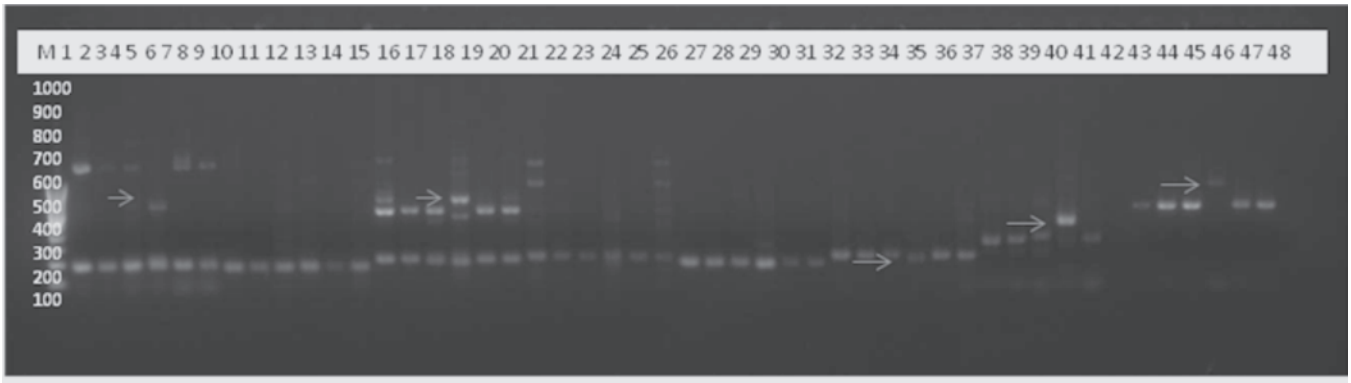

Fig. 4. Lane 1 DNA Ladder 100bp (Fermentas), Lane 2-48, genotypes in sequence (WIR 3928, Punjab Chhuhara, H-88-78-1, EC-520061, CO-3 and Sel-7). Primers (SSR 226, SSR 210, SSR 308, SSR 310, SSR 316, SSR 124, SSR 112 and SSR 356)

2003). The conditions (average temperature 22 ºC and humidity around 90%) which favour the disease are prevalent in the study area. A major hindrance in breeding tomatoes for early blight resistant has been the screening process due to its limitations such as environmental conditions. Field screening can often be carried out only once a year, such limitations would restrict breeding process (Foolad *et al.*, 2000). The effectiveness of each method may vary by species to species and by application. Therefore, it is reasonable here to use more than one method. In the present study, PDI, AUDPC and r were used to evaluate and compare disease severity of tested tomato lines. Periodic observations of PDI are essential to assess the pathogenic reaction on a particular line. Based on a single observation, it is difficult to evaluate the disease severity and pathogen reaction of the same line at later stages (Pandey *et al.*, 2003). The present findings also confirm that the PDI and AUDPC are important for controlled environment and apparent infection rate are important for natural screening programmes. The disease severity initially progress slowly but accelerate as plant matures. The highly resistant and resistant lines EC-520061 (*S. habrochaites*), EC-521080 (*S. pimpinellifolium*), WIR-3928 (*S. glandulossum*) and H-88-78-1 (*S. esculentum*) have indeterminate growth and according to Pandey *et al.* (2003), these resistance lines are stable and an indication of resistance against early blight disease caused by *A. solani*.

An area under disease progress curve which refers to as the signature of an epidemic, represent the integration of all the host, pathogen and environmental factors during epidemics (Campbell and Madden, 1990). Screening under artificial condition is more informative than natural conditions and for inoculation only mycelia cultures of the pathogen were used. The result confirms that the degree of susceptibly to *A. solani* infection increases with the age of plants and the influence of climatic factor is minimal because of the controlled condition. In inoculated plants, disease incidence always increased during the initial disease development (7-28 days after inoculation) and thereafter subsequently up to 90 days. Disease developed quickly during natural epidemics at the time of fruit set and infected leaves quickly defoliated. It further increased if either irrigation or rain prevailed in the field. Similar results were recorded on tomato (Pandey *et al.*, 2003; Upadhyay *et al.*, 2009) and other crops (Rotem, 1994; Pandey and Pandey, 2002). Our study has established that the parameters *e.g.*, PDI, r and AUDPC are important and useful to screen tomato lines against early blight caused by *A. solani*.

Many PCR based tomato markers have been developed and mapped, and are publicly available from a variety of sources (*e.g.* http://www.sgn.cornell.edu/, http://hornbill.cspp.latrobe.edu.au/ssrdiscovery.html). These resources enabled screening a set of genome-wide markers to identify polymorphic markers distinguishing genotypes previously screened in field and glasshouse test (artificial screening). If the markers are informative, they may be also applied on the population developed between the crosses. (resistant vs. susceptible). The selected primers are useful for QTL mapping against early blight reaction. Majority of these anchor markers such as SSR 108 (500bp) which exhibited polymorphism in the survey had previously associated with early blight mapping (Chaerani, 2000) indicating their usefulness for mapping in other populations also.

It is notable in the present findings that cultivated tomato line H-88-78-1 (*S. esculentum*) performed equally well as wild relatives, EC-520061 (*S. harbochaites*) in suppressing the disease. In view of the above, it is concluded that the genotypes EC-520061 and H-88-78-1 may be used as donor parents to develop early blight disease resistance/tolerant cultivars/hybrids and could be of immense importance for further traditional or molecular breeding programs.

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