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Comparison of Detached Leaf Evaluations in Different Age of Transgenic Tomato Expressing Antimicrobial Peptide Gene (*Ace-AMP1*) Resistant to Early Blight Disease Caused By *Alternariasolani*

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ABSTRACT: Tomato cultivar Arkavikas were transformed with *Ace-AMP1* gene, regenerated plants were screened for the presence of gene and T1 transgenic leaves were used for detached leaf bioassay evaluations. Three different stages of transgenic tomato leaves were evaluated for resistance to early blight caused by the fungus *Alternariasolani*. Evaluations were conducted in three replications in growth chambers using detached leaves under controlled conditions. Leaf samples were plated on sterile blotting paper with 3 mm fungal plug along with respective control. Percentage of lesion diameter were calculated and compared with control for disease resistances. Forty day old leaf developed chlorotic lesions in 3 days and were more susceptible to disease when compared to 60 and 90 day old leaves and the correct age for detached leaf bioassay evaluation is 90 day old leaf. Transgenic line 10-7 was highly resistant towards *Alternariasolani* and moderate levels of resistance were observed in 7-2, 13-19 and 1-3. The detached leaf bioassay offers an early, compact and relatively fast method to detect resistance levels in transgenic plants when compared to other screening methods.

KEYWORDS: Early blight disease, Transgenic tomato, Antimicrobial peptide gene, Leaf bioassay, Disease resistance.

I. INTRODUCTION AND BACKGROUND

Tomato (*Lycopersicmesculentum*L.spp) is the most important vegetable crop cultivated worldwide with a high nutritive value, India the second top producers of tomatoes with an annual production of 17,500,000 tonnes (FAOSTAT, 2012). Tomato plants are highly suspectible to various diseases like viral, bacterial, fungal and nematode disease. Early blight (EB) is the most common disease of tomato caused by *Alternariasolani*, affects the yield and reduces fruit quality. EB is a serious problem in warm and humid regions [1] and also in semiarid areas. EB reduces the photosynthetic area, and defoliates the lower parts of leaves, it forms brown spots on leaf, fruits and stems, if uncontrolled early the entire plant collapses. An attempt was made to genetically modify the crop with antimicrobial peptide gene (*Ace-AMP1*) which brings aboutcytoplasmatic membrane disruption [2] [3] and also can inhibit several cellular processes such as nucleic acid and protein synthesis, enzymatic activity and cell wall synthesis [4] [5] [6] [7] [8. Transgenic plants were developed for resistance towards early blight disease. There are several techniques employed to screen transgenic plants resistant towards a disease. The normal way of screening for disease is by field and greenhouse screening which is time consuming, affected by various external environmental factors such as temperature, humidity and influence of other pathogens in the study. These methods require challenging of plants with pathogens and there is a possibility of contaminating the atmosphere thus resulting in spread of disease to all the healthy plants. In view of all the most efficient and simpler attempt is by the detached leaf bioassay, with correct aged leaf in a laboratory condition, thus

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contaminating the atmosphere is nil. In tomato detached leaf bioassay was first carried out by Douglas, [9] to compare the disease resistance. Later this methodology was used to compare the virulence of different *Altenariasolani* for evaluating the resistant genotypes to be either ineffective [10] or effective [11].

II. MATERIALS AND METHODS

i. Raising of putative transformed tomato plants

Seeds of tomato cultivar Arkavikas were inoculated and hypocotyledons were excised from 8 day old seedlings. T0 transgenic tomato plants were generated with pCAMBIA 2301 carrying Antimicrobial peptide gene (*Ace-AMP1*), with the constitutive expression of cauliflower mosaic virus (CaMV 35S) promoter, NOS terminator and nptII as a selection marker. Regenerated plants were screened for the presence of gene by PCR and T1 plants were raised from T0 seeds. T_1 transgenic tomato plants which were southern positive were selected for detached leaf bioassay.

ii. Inoculum preparation for leaf bioassay

An *Alternariasolani*isolate was obtained from infected tomato leaves in cultivar Arkavikas, IIHR, Bangalore, it was propagated on potato dextrose agar (PDA) in 90-mm-diameter petri dishes and in 10 days of incubation the culture covered the entire plate as a matte. The culture was sub-cultured on PDA 3 – 4 days prior to use, plugs of *Alternariasolani* were formed with a sterile #1 cork borer (~ 3 mm diameter) around the perimeter of actively growing culture just before inoculation.

iii. Age of leaf and plant material treatment

Age of leaf was determined form the day of seed sowing. Three stages of leaves were used to determine the effect of leaf age on detached leaf bioassay I) Leaves were isolated form 40 day old portray seedlings. II) Terminal leaflet from 2 month (60 day) old plant and III) The third oldest leaf from 3 month (90 day) old plant were used for detached leaf bioassay. All the leaf samples were collected, the leaves were treated with 70% ethanol followed by 1% sodium hypochlorite wash for 2 mins and the samples were washed with sterile distilled water to remove all the traces of sodium hypochlorite and blotted on a tissue paper. The petiole of the all the leaves were covered with moist cotton to avoid leaf drying. Sterile blotting paper was cut into the required size of petridishes and moistened with sterile distilled water. Fungal plug containing *Alternariasolani* were placed in centre on the adaxial side of leaf along with control leaf (devoid of fungal plug). Petridishes were moistened with sterile distilled water every 48 hrs. The plates were incubated in room temperature and all the experiment was carried out in three replications. Observations were taken from third day and lesion diameter was recorded. In the end of observation a post hoc analysis was carried out by inoculating the infected leaf on PDA media.

iv. Statistical analysis

Data from all the sets of detached leaf assay experiments were recorded in three replicates and subjected to analysis of variance (ANOVA). Total area of green colour and chlorotic lesion area was measured to calculate the deterioration of detached leaves. Percentage of infected leaf area for transgenic and control leaves was measured using ImageJ a Java-based image processing tool. A post hoc analysis test LSD (Least Significant Difference) was carried out in disease assay experiment and to test the significance of different treatment means against a standard control in accordingly presented in tables. All analysis were done using GraphPad Prism-5 a statistical package tool.

III. RESULTS

*Alteranariasolani*culture started growing on PDA media in 3 days, in 7 – 10 days the culture grew upto the periphery of petridish. Surface sterilization of leaves helped to remove the surface bacteria and fungi to a greater extent. Placing the leaves on moistened filter paper and maintaining moist throughout the experiment helped in avoiding the leaf from senescence. All the control leaves which were inoculated with a plug of *Alteranariasolani*turned completely brown and un-inoculated control and un-inoculated transgenic leaf remained green. Chlorosis with distinct differences varied for all the three experiments and the lesion measurements were taken accordingly. Percentage of lesion diameter was high on 40 (Fig.1a) day old leaf when compared to 60(Fig. 1b) and 90 day (Fig.1c) old leaf. On 40 day old leaf, lesion development was observed within 3 days of inoculation and in 12 days the entire leaf turned brown when compared to the other experiments.

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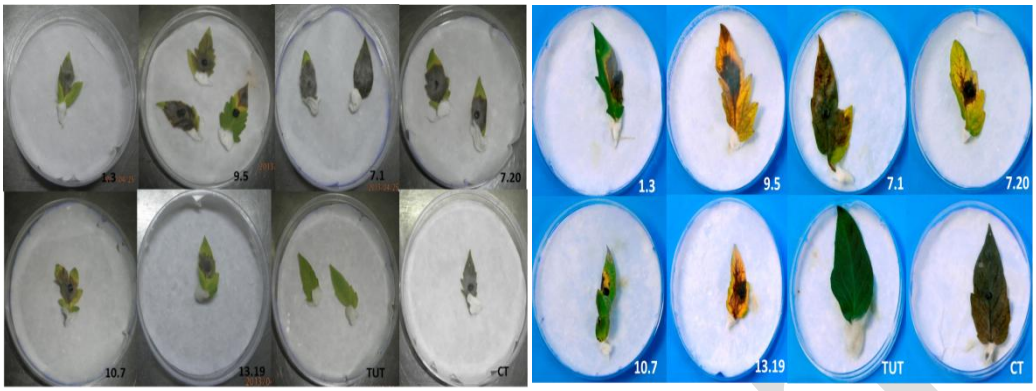
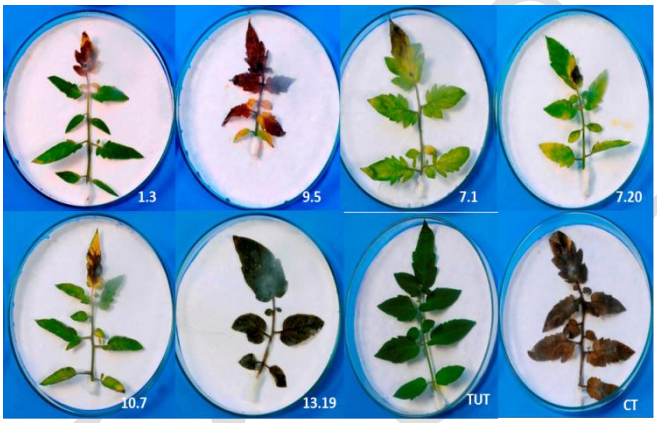


(a) (b)



(C)

Fig. 1. Comparision of detached leaf bioassay on 40, 60 and 90 day old onTransgenic leaves inoculated with *Alternaria solani*. (a) 40 day old, (b) 60 day old, (c) 90 day old detached leaf bioassay. Transgenic line – 1-3, 9-5, 7-1, 7-20, 10-7, 13-19. CT – Control treated, TUT – Transgenic uninoculated.

In 40 day old leaf experiment, the leaf samples are too young and increased the susceptibility towards disease in all the lines of transformants. Transgenic line 10-7 was highly resistant towards *Alteranariasolani* when compared to the other lines. Transgenic line 7-2, 13-19 and 1-3 showed moderately lower levels of chlorosis (Table: 1) and resistant towards the disease. Transgenic line 1-3 and 7-1 were non-significant on 40 day old detached leaf bioassay but in 90 day old leaf assay they were significant. Differences of results were observed in all the treatments, as the age of leaf were older the resistant levels also increased, in younger leaf stage the susceptibility towards the disease is higher. So selecting a correct age of leaf is a crucial point in detached leaf bioassay. Post hoc analysis on PDA media further confirmed that the lesion development was caused by *Alteranariasolani.*

Table 1:Detached leaf assay of control and transgenic tomato lines of *Ace-AMP1*

Control / Transgenic lines	% of diseased leaf area		
	40 day old leaf	60 day old leaf	90 day old leaf
CUT	0	0	0
TUT	0	0	0
CT	100	100	100
1-3	74.86 ± 9.6	20.78 ± 6.60 ***	16.23 ± 8.25 ***
9-5	34.52 ±10.29 ***	33.4 ± 20.31 ***	22.23 ± 8.78 ***
7-1	72.05 ± 5.22	47.05 ± 24 *	45.48 ± 2.06 ***

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7-2	64.91 ± 1.88 *	14.98 ± 2.83 ***	9.372 ± 5.70 ***
10-7	7.869 ± 2.81 ***	4.986 ± 2.19 ***	1.747 ± 1.04 ***
13-19	39.57 ± 5.65 ***	37.55 ± 7.92 **	12.51 ± 3.76 ***

Different age of leaf on detached leaf bioassay of Transgenic tomato expressing *Ace-AMP1* gene against *Alternariasolani.* Data represents the percentage of lesion development and measured using ImageJ software. The significance of difference between mean values was evaluated by LSD post hoc test. *, ** and *** indicates $P < 0.05$, 0.01 and 0.001 respectively.

IV. DISCUSSION

Screening for resistance for transgenic plants is generally conducted in field conditions which have limitations like; suitable environmental conditions, inhibitions from other pathogens, longer duration to observe results [12]. It also requires approval from various governing committees like RCGM and GEAC to conduct experiments in field condition. The detached leaf bioassay overcomes the limitations of time and space, provides a compact environment, relatively fast and the most reliable assessment for early screening of transgenic plants against *Alternariasolani*. Selection of Leaves should be devoid of mites and other pathogens, which might obstruct the accurate results in detached leaf bioassay. Sterilization of leaf samples reduced the saprophytic populations and similarly [13]. Antimicrobial peptide genes have high antimicrobial activity against various pathogenic fungi and are the potential genes to be transformed into plants for transgenic development [14] [2]. In the present study, transgenic plant 10-7 showed higher resistant and moderate resistant was observed in 7-2, 13-19 and 1-3 when compared to the other lines. Wu *et al*[15] applied Ace – AMP1 protein on the leaves of tomato and infected with *Alternariasolani* which showed strong resistance towards the pathogen.In this experiment it was found that older leaves 90 days were more resistant towards the disease when compared to younger leaves. The same was observed with [16] that the chickpea older leaves were more resistant than the younger leaves. In contrast Browne *et al*[17] reported that detached leaf 3 (youngest leaf) was more resistant than leaves 1 and 2. Selecting leaf of similar age and position on the plant is an important factor in detached leaf bioassay for consistent results [18]. Ninety day old leaf is better in performing detached leaf bioassay.

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