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***Research Article***

# Resistance analysis of four Chickpea cultivars against fusarium wilt using biochemical markers

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**Abstract**

*Fusarium* wilt is one of the major constrain in the Chickpea production. Present study is focused on visual screening of four Chickpea cultivars against fusarium wilt and its biochemical correlation with PR proteins and Phytoalexin. From the field experiments, it was observed that, out of four cultivars selected for the study, Digvijay, Vijay and Jaki were found to be highly resistant, resistant and moderately resistant/tolerant with fusarium wilt incidence 7%, 12% and 34% respectively while JG-62 was found to be susceptible with disease incidence 38%. This disease incidence data was correlated with level of biochemical markers like PR proteins and phytoalexin. It indicates the positive correlation, as level of both PR proteins (glucanase and chitinase) and phytoalexin medicarpin was highest in cultivar Digvijay, followed by Vijay and Jaki and least in JG-62.

## INTRODUCTION:

Chickpea (*Cicer arietinum* L.) is one of the most important food legumes grown worldwide, especially in dry land areas of the Indian subcontinent (Saxena, 1990). In the European Union, chickpea production is restricted to the Mediterranean Basin, with Spain being the principal producer. *Fusarium* wilt, caused by *Fusarium oxysporum* f.sp. *ciceris* (Foc), is a major constraint to chickpea production worldwide (Jalali and Chand, 1992). Annual chickpea yield losses from *Fusarium* wilt vary from 10 to 15% (Trapero- Casas and Jiménez-Díaz, 1985; Jalali and Chand, 1992), but the disease can completely destroy the crop under specific conditions (Halila and Strange, 1996). The most effective and practical method for management of the disease worldwide is the use of resistant cultivars (Jiménez-Díaz *et al* ., 1991; Jalali and Chand, 1992; Kraft *et al* ., 1994). However, the effectiveness of host resistance is curtailed by the occurrence of other pathogenic races.

The use of resistant cultivars for management of fusarium wilt in chickpea may be enhanced by means of biological control using either bacterial or fungal antagonists. The objective of this research was to determine the roles of biochemical compounds, in the interaction of chickpea with *F. oxysporum.*

## MATERIALS AND METHODS:

**Germplasm Procurement and field experiment:** The germplasm of four Chickpea cultivars (Vijay, Digvijay, Jaki and JG-62) was procured from Dr. P. D. K. V. Akola and M. P. K. V. Rahuri (MS). The procured seed material of each cultivar was screened out for damaged seed structure and normal seeds are used for further sowing in field and in vitro experimentation. 500 seeds of each cultivars were sown in well prepared plots separately. Each plot was observed for germination, disease incidence, mortality due to environmental stress, days to flower and maturity.

**Disease incidence and Severity analysis**

In all experiments, disease incidence (percentage) and severity were assessed at 2-day intervals. Severity of symptoms on individual plants were rated on a scale from 0–4 according to the percentage of foliage with yellowing or necrosis in acropetal progression: 0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, 4 = dead plant. Incidence and severity data (0–4 scale) within a pot were used to calculate a disease intensity index (DII) by the equation: DII = [(ΣSi x Ni)/ 4x Nt) x 100]

Where, *S*i = symptom severity; *Ni*= number of plants with *N*t= total number of plants. Thus DII expresses the mean value of disease intensity at a given moment as a proportion of the maximum possible intensity.

**Extraction and PR-protein assay**

Chitinase -1,3-glucanase were extracted by homogenizing 1g of frozen tissues in 1 ml and ice cold extraction buffer (0.1M Sodium citrate buffer, pH 5), in pre-chilled mortar and pestle. The extract was centrifuged at 10,000 rpm for 15 min. 1ml of supernatant was taken in 1.5 ml centrifuge tube and proteins were precipitated by adding ammonium sulphate to saturation. The precipitated protein were centrifuged at 10,000 rpm for 15 min and supernatant was discarded. The protein pallted resuspended in 1ml extraction buffer and used as enzyme source for glucanase and chitinase activity.

The assay of β-1,3 glucanase was performed according to method given by Kauffmann et al., (1987). The assay mixture was prepared by mixing 0.48 ml of 01M Sodium acetate buffer (pH, 5.2), 100 µl of enzyme extract, and 200 µl Laminarin (Sigma) solution. The mixture was incubated at 37$^{o}$C for 3 hrs. Then, 0.5 ml alkaline copper tartarate was added to it and mixture was heated at 100$^{o}$C, in the boiling water bath for 5 min. The mixture was then cooled to room temperature and 0.5ml of arseno-molybdate reagent was added to it. After the development of blue color, 3ml of distilled water was added to each sample and absorbance was recorded at 660 nm against the blank containing the enzyme extract and all other reagent except Laminarin.

The chitinase activity was analyzed according to the method of Reissig *et al*., (1954) and Boller *et al*., (1983). The enzyme assay mixture contained 100 µl of sodium acetate buffer (pH 4.5), 100µl Sodium azide solution, and 200 µl colloidal chitin and 100 µl enzyme extract. The volume of this enzyme extract was adjusted to 1 ml by extraction buffer and incubated at 37$^{o}$C for 3 hrs. Then 100 µl of Sodium borate buffer (pH 9.1) was added to it and heated to 100$^{o}$C in water bath for 3 min. The mixture was then cooled in tap water and centrifuged at 1000 rpm for 5 min. The clear supernatant was collected and to it 3 ml of DMAB reagent was added. The mixture was then incubated for 20 min at 37$^{o}$C. The absorbance was recorded immediately at 585 nm against the blank containing all the reagents and enzyme except chitin.

**Phytoalexin analysis**

The level of phytoalexin Medicarpin was analyzed in the leaves of naturally infected field grown plants. For this the leaves of plants from each cultivar were collected 10, 20, 40, 60, 80 days after germination. The collected leaves were frozen in liquid nitrogen and then stored in -20oC, until use. One gram of leaf sample was extracted in 5ml 80% methanol. The methanol extract was reduced to 1/4$^{th}$ of initial volume under vacuum and extracted (3x) with ethyl acetate. The pooled ethyl acetate extract was reduced to dryness and dried residue was dissolve in l ml acetonitrile. The 20 µl acetonitrile extract was injected to quantify medicarpin. The samples were chromatographed on Shimadzu HPLC system with ODS $C_{18}$ (Spherosphere) column (4x250 size) maintained at 35oC temperature. The flow rate of mobile phase (50% aqueous acetonitrile) was 1.50 ml/ min. The medicarpin was detected at 290 nm using PDA detector with retention time 23 min.

**RESULTS AND DISCUSSION**

The field experiment reveals that Chickpea cultivar Digvijay is highly resistant against fusarium wilt and showed only 7% disease incidence. The disease incidence percentage of cultivar Vijay and Jaki was found to be 12% and 34% respectively indicated that both cultivars are resistant to fusarium wilt with Vijay falling in resistant and Jaki in moderate resistance class on the basis of disease incidence index. However, the cultivar JG-62 was with 38% disease incidence index and considered as susceptible to fusarium wilt (table-1). The phytoalexin accumulation in the leaves of naturally grown population after 60 days after germination, cultivar Digvijay showed highest values followed by in leaves of cultivar Vijay and Jaki, while phytoalexin value is least in cultivar JG-62 (table-2). Similar trends of accumulation of PR proteins chitinase and b-1,3 glucanase was observed (Fig. 1). Thus, the level and accumulation of phytoalexin

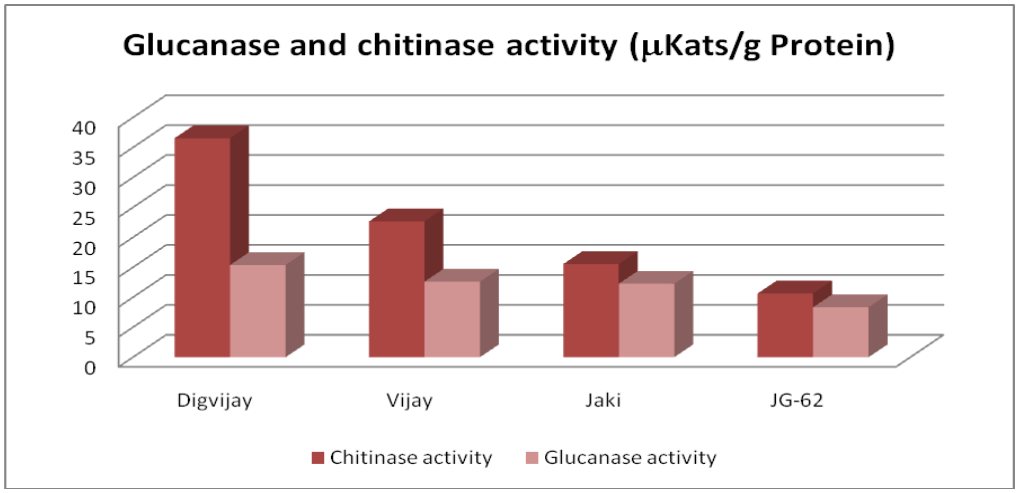
**Table-1 Disease incidence and disease severity of *Fusarium* wilt in four naturally grown cultivars**

| Cultivar | Progeny size (No. of plants) | Disease incidence | Resistant status |
|---|---|---|---|
| Digvijay | 480 | 07% | Highly Resistant |
| Vijay | 460 | 12% | Resistant |
| Jaki | 430 | 34% | Moderate Resistant |
| JG-62 | 468 | 38% | Susceptible |

**Table-2: Medicarpin analysis in leaves of naturally grown Chickpea cultivars**

| Cultivar | Phytoalexin Medicarpin content (µg/g) in leaves (days after germination). | | | | |
|---|---|---|---|---|---|
| | After 10 days | After 20 days | After 40 days | After 60 days | After 80 days |
| Digvijay | 10.6 | 24.7 | 45.6 | 81.5 | 52.3 |
| Vijay | 09.2 | 21.6 | 42.9 | 75.3 | 48.7 |
| Jaki | 09.9 | 16.3 | 29.5 | 17.2 | 15.6 |
| JG-62 | 08.5 | 18.5 | 22.9 | 13.8 | 15.4 |

**Fig. 1. Glucanase & Chitinase activity in leaves of Naturally grown plants of chickpea cultivars (µkats/mg protein) 60 days after germination**




and PR-proteins in plant could be positively correlated with the disease incidence index in case of Chickpea- fusarium wilt pathosystem. Similar correlation was reported by Cachinero *et al.* (2002) in three Chickpea cultivars. Koche and Choudhary (2005) also reported that the level of PR- protein in Mungbean cultivars could be positively correlated with its resistance status. In 2007, Badere *et al.*, reported the positive correlation of phytoalexin accumulation and resistant status of seven mungbean cultivars. These findings are also supported by the reports of Koche and Choudhary (2011). Balerao and Kothekar (2013) have reported the positive correlation between resistance of crop with its biochemical content. Khandare (2015) demonstrated that, the plants with pathogen attack has less protein content. The results of the present study are in accordance with above reports and could be exploited further at molecular level for developing fusarium wilt resistant Chickpea cultivars.

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