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Effect of native *Trichoderma viride* and *Pseudomonas fluorescens* on the development of *Cuscuta campestris* on chickpea, *Cicer arietinum*

C. Kannan*, B. Kumar, P. Aditi[1] and Y. Gharde

Directorate of Weed Science Research, Maharajpur Jabalpur- 482004 (M.P), INDIA
[1]Department of Biological SciencesRani Durgavati University, Jabalpur- 482001 (M.P), INDIA
*Corresponding author E-mail: agrikannan@gmail.com

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Abstract: *Cuscuta campestris* Yuncker is a serious parasite on several leguminous crops including chickpea in India. Chickpea is an important pulse crop in India and severe incidence of *Cuscuta* may result in yield loss of about 85.7%. Management of *Cuscuta* is very difficult because of their intricate relationship with the host, wide host range and lack of resistant genes in the host. Thus induced systemic resistance (ISR) by plant growth promoting microbes (microbial elicitors) may be an effective alternative method for the management of *Cuscuta*. In the current study, to induce systemic resistance, native isolates of *Trichoderma viride* Pers. and *Pseudomonas fluorescens* Flügge were used as seed treatments and foliar spray on chickpea and then infested with *C. campestris*. Salicylic acid and thiobenzamidazole (synthetic elicitors) were used as standard inducing agents for comparison. Results indicated that fresh seeds of *C. campestris* germinated rapidly even without scarification and that the germination was not influenced by the proximity of the seeds to the host. Seed treatment followed by foliar sprays with the bioagents and synthetic elicitors induced at 20 and 40 days after sowing (DAS) induced increased production of defense enzymes in chickpea (*Cicer arietinum* L.) and thus delayed the development (1.8-5 days) and flowering (2.4-4.2 days) of *C. campestris*. Treatment with both the elicitors also resulted in the enhanced activities of scavengers of enzymes related reactive oxygen species (ROS). Thus the above work would help in the integration of the application of bioagents for effective management of *Cuscuta* in chickpea.

Keywords: Chickpea, *Cuscuta campestris*, Defense enzymes, ISR, *Pseudomonas fluorescens, Trichoderma viride*

INTRODUCTION

Cuscuta spp. *(*commonly called as Dodder*)* are rootless, achlorophyllous, heterotrophic, obligate angiosperms twining on dicotyledonous crops. They belong to the family Cuscutaceae (earlier known as Convolvulaceae), containing about 170 different species distributed throughout the world (Holm *et al.*, 1997). *Cuscuta* are broadly nonspecific, attacks a wide range of plant species including many cultivated plants and dicotyledonous weeds, but rarely the monocotyledonous plants (Wright *et al.,* 2011). *Cuscuta* enjoys a very intimate relationship with the host plants throughout its life cycle, except for a very short, post germination independent period of about 8-10 days, in which even a two way transfer of genes between host and the parasite has been reported (Mower *et al.*, 2004).

Among the 12 different species of *Cuscuta* reported to occur in India, *C. campestris* and *C. reflexa* are most common and cause significant economic losses on crops like niger, lucerne, berseem and chickpea (Gaur, 1999). Incidence of *Cuscuta* spp. is reported mainly in the states of Andhra Pradesh, Chhattisgarh, Gujarat, Odisha, West Bengal and Madhya Pradesh on oilseed crops like niger, linseed, pulses viz., blackgram, greengram, lentil, chickpea (prominently in rice-fallows) and fodder crops including lucerne, berseem (Mishra, 2009). Chickpea is an important pulse crop, cultivated in about 8.56 million ha with an annual production of 7.35 million tones and India is the largest producer, accounting for nearly 64% of the global production (Gaur *et al.*, 2010). Vyas and Joshi (1975) first reported the incidence of *Cuscuta* sp on chickpea in the state of Uttar Pradesh, in India. Mishra (2009) reported that *C. campestris* is the dominant species attacking chickpea in India. Yield loss of about 85.7 % has been reported in chickpea as a result of *Cuscuta* infestation (Moorthy *et al.*, 2003) and 54.7 to 98.7 % by 1-10 *C. campestris* twines /m^2 (Mishra, 2009).

The choice of chemicals for control of *Cuscuta* is very limited. Pre-plant incorporation and post emergence application of imazethapyr at 75 g/ha produced better control of the *Cuscuta* on various crops (Mishra *et al.*, 2007). Inherent genetic resistance in the host against *Cuscuta* is not very common (Lanini and Kogan, 2005) and crop rotation is not a feasible technique often because of its wide host range. Thus induced systemic

resistance (ISR) by microbes is thought of as an integrated strategy in the management of *Cuscuta*. Plant growth promoting microbes induce resistance in plants by activation of host anti-stress genes to produce more defense proteins and phytoalexins against plant pathogens (Van loon *et al*. 1998; Kannan and Karthik, 2009; Sriram *et al.,* 2009), alter the composition of host root exudates and their volatile signaling molecules (Harsh *et al.*, 2006), thereby interfering with the recognition of the host by the parasite. *Cuscuta* resembles the plant pathogenic fungi in the use of haustoria as the main invading organ to infect and establish in the host (Meyer, 2006) and thus would fit well in the scheme of management by ISR. Keeping this in view, the present investigation was conducted to study the effect of native *Trichoderma viride* and *Pseudomonas fluorescens* on the development of *Cuscuta campestris* on chickpea (*Cicer arietinum).* This study would help in the integrated management of *Cuscuta* by means of application of microbes at appropriate stages of cultivation. Further since the awareness about the ill effects of more usage of pesticides is increasing, this safe and natural method of management using friendly microbes would be of significant importance in the overall strategy for the management of this dreaded weed.

MATERIALS AND METHODS

Location: Experiments were conducted in controlled conditions in the containment facility at the Directorate of Weed Science Research (DWSR), Jabalpur ($23^0$13'59.00"N, $79^0$58'02.25"E, elevation 390.45m) during 2009 to 2012. *C. campestris* seeds were collected from the farmer's fields in Mandla district of Madhya Pradesh ($23^0$31'51.54"N, $80^0$27'55.49"E, elevation 456.60 m).

Antagonistic microbes: Fungal and bacterial bioagents were isolated from native soils of chickpea using appropriate selective media *viz., Trichoderma* selective medium (Elad *et al.*, 1981) for *T. viride* and King's B for *P. fluorescens*. To prevent attenuation the bioagents were periodically inoculated in the pots with chickpea infested by *C. campestris* and again reisolated.

Effect of antagonists and synthetic elicitors on *Cuscuta C. campestris* on chickpea: Plastic tubs of size 30 cm^3 were filled with pot mixture containing sterilized soil, sand and decomposed (Farm Yard Manure**)** FYM (1:1:1). Locally popular variety of chickpea, JG-16 was sown and seedlings were thinned to maintain 2 healthy seedlings per pot. Seeds of *C. campestris* was sown by thoroughly mixing about 20 seeds with the top soil of the pot and with a rose can, watered gently using tap water (EC = 2 ds/m, pH= 7.1).

Antagonists were multiplied in their respective broths, 6 days (*P. fluorescens*) and 10 days (*T. viride*) by incubating at 30 ^{0}C in a shaking incubator, after which the broth solution along with the microbial mat was collected, homogenized in a blender and applied as foliar spray or used for seed treatment. Synthetic elicitors 0.5M *viz.*, salicylic acid and thiobenzamidazole (Bion 50% obtained from M/s Syngenta India Ltd.) were similarly used for comparison. Chickpea treated with distilled water was maintained as control.

Antagonists- chickpea-*Cuscuta* interactions: To study Induced Systemic Resistance (ISR) in chickpea, the potted plants were treated with the bioagents and infested with *Cuscuta*. The treatments with five replications per treatment are given as follows:

T_1:	Seed treatment with *P. fluorescens*
T_2:	Seed treatment with *T. viride*
T_3:	Seed treatment with salicylic acid (0.05M)
T_4:	Foliar spray with *P. fluorescens* at 20 DAS and 40 days
T_5:	Foliar spray with *T. viride* at 20 DAS and 40 days
T_6:	Foliar spray with salicylic acid (0.05M) at 20 DAS and 40 DAS
T_7:	Negative control (Chickpea+*Cuscuta)*
T_8:	Control (only chickpea)

Activity of five key defense enzymes viz., Chitinase (CH), Catalase (CT), Poly Phenol Oxidase (PPO), Peroxidase (PO) and Phenylalanine Ammonia Lyase (PAL) were estimated from the stem tissues of young plants collected from the above treatments periodically viz., immediately after the spraying (0 day), and further upto 50 days at an interval of 10 days from application, when the enzyme activity became static or declines. Colorimetric assay of enzyme CH was carried out according to Boller and Mauch (1988). PAL activity was estimated as described by Dickerson *et al.* (1984). The enzyme PO was analysed as given by Hammerschmidt *et al.* (1982), CT according to Aebi (1983) and PPO according to Meyer *et al.* (2000). To study the activity of the antioxidant enzymes like superoxide dismutase (SOD), Glutathione Reductase (GR) and Glutathione Peroxidase (GPX) both in chickpea and *C. campestris*, the samples were drawn from the above experiments and analyzed. The SOD activity was estimated using xanthine-xanthine oxidase system as suggested by Beyer and Fridovich (1987). The enzyme GPX was assayed as per the method suggested by Inoue *et al.* (1999).

Statistical analysis: All the experiments were conducted in Randomized Block Design (RBD) for two consecutive years and since there were no significant interactions between observations, the data were combined over the years and subjected to analysis of variance (ANOVA). Regression analysis was used where appropriate: Otherwise means were separated using least significant difference (LSD) at 5% level of significance. Before the analysis,

normality of data and the equality of variances were checked using Kolmogrov-Smirnov test and some variables were transformed using suitable transformation. ANOVA was performed on data using general linear models procedure using PROCANOVA procedure with the SAS 9.2 statistical software (SAS Institute Inc., USA). Significant differences between different treatments were observed using Tukey's Honest Significant Difference. Linear model was best fitted to the flowering in *Cuscuta* at different distance from host plant chickpea. The model is given as Y= a+bx, where, a and b are the regression coefficients of the model and y and x represents the flowering in *Cuscuta* and distance of *Cuscuta* from the chickpea, respectively.

RESULTS AND DISCUSSION

Germination, host search and development of *C. campestris:* From the above study, it was observed that *C. campestris* germinated within a period of 3-4 days after sowing without acid scarification, when the fresh seeds were used. Germination of *C. campestris* was not influenced by the distance of its seed to the host seedling (Table 1) and there was no significant difference in the percentage germination of *C. campestris* when sown at different distances from the host plant. However treatment of chickpea with the bioagents or the synthetic elicitors influenced the germination of the *C. campestris* seeds and also affects the number of days taken by *C. campestris* to establish in the host and initiate flowering (Table 2, Fig. 1). Bion, when applied as seed treatments caused maximum delay in establishment of *C. campestris* by 16.4 days (30.50% over control) when sown at 12 cm away from the host, followed by salicylic acid 10.6 days (28.3% over control) when sown at 6 cm away. Among the bioagents, *P. fluorescens* was able to delay the process of establishment by 10.42 days (26.92% over control). However, when compared for the days taken to first flowering by *C. campestris*, which indicates the development and physiological maturity of the parasite, *P. fluorescens* was found to cause maximum delay of 25.20 days (29.36% over control)

Table 1. Effect of seed treatment and folia spray of bioagents and synthetic elicitors on germination and host search of *Cuscuta* in chickpea**.**

Treatments	Cuscuta infecting chickpea (DAS)				Flowering in *Cuscuta* (DAS)			
	3 cms	6 cms	9 cms	12 cms	3 cms	6 cms	9 cms	12 cms
Seed treatment followed by foliar spray with *T. viride* at 20 DAS and 45 days	6.80ab	9.00bc	12.40a	13.20c	20.40c	22.00ab	26.60c	29.40b
Seed treatment followed by foliar spray with *P. fluorescens* at 20 and 45 DAS	7.00ab	10.40ab	13.40a	14.40bc	25.20a	23.00a	28.80a	31.00ab
Foliar spray with salicylic acid (0.05M) at 20 DAS and 45 DAS	7.40a	10.60a	12.80a	14.80b	23.20b	22.80a	27.20 bc	29.80ab
Foliar spray with Bion (0.05M) at 20 DAS and 45 DAS	8.00a	10.80a	13.40a	16.40a	24.00ab	23.40a	28.40 ab	31.20a
Chickpea+*C.campestris* (control)	5.80b	7.60c	9.80b	11.40d	17.80d	20.40b	22.20d	27.00c
LSD @0.05	**1.20**	**1.40**	**1.38**	**1.40**	**1.39**	**1.85**	**1.33**	**1.78**

Table 2. Linear model fitting of data on flowering in *C. campestris.*

Treatments	Coefficient estimates		R2
	a(SE)	b(SE)	
Seed treatment followed by foliar spray with *T. viride* at 20 DAS and 45 days	16.70 (1.06)	1.05 (0.13)	0.97
Seed treatment followed by foliar spray with *P. fluorescens* at 20 and 45 DAS	20.10(0.54)	0.92 (0.07)	0.99
Foliar spray with salicylic acid (0.05M) at 20 DAS and 45 DAS	19.5(1.36)	0.83(0.17)	0.92
Foliar spray with Bion (0.05M) at 20 DAS and 45 DAS	19.80(1.41)	0.93(0.172)	0.94
Chickpea + *C.campestris* (control)	14.5(1.20)	0.98(0.15)	0.96

Table 3. Studies on the activity of five defence enzymes upon treatment with the bioagents and the synthetic elicitors.

Enzymes	**Days of observation after treatment**											
	Phenylalanine ammonia lyase (PAL)						**Chitinase (CHI)**					
Days	**0**	**10**	**20**	**30**	**40**	**50**	**0**	**10**	**20**	**30**	**40**	**50**
T_1	2045.00f	2107.25f	2410.75f	2213.50f	2188.00f	2478.25e	2249.00b	2305.75b	2395.25b	2353.50b	2346.75b	2410.00b
T_2	2173.75e	2274.25e	2449.25e	2417.50e	2407.25e	2477.25e	2211.00c	2249.00c	2357.00c	2305.75c	2304.50c	2398.75c
T_3	2242.50d	2312.50d	2515.50d	2455.00d	2455.75d	2579.50d	2016.25d	2059.00d	2230.50d	21.02.50d	2091.50d	2261.25d
T_4	2951.25b	3017.00b	3228.00b	3114.75b	3011.25b	3247.50b	1848.25f	1902.75f	2005.00f	1957.50f	1951.50f	2055.00f
T_5	2648.75c	2710.00c	2943.75c	2850.75c	2811.50c	2943.50c	1971.75e	2005.00e	2102.50e	2051.00e	2044.25e	2155.00e
T_6	3104.50a	3211.25a	3520.50a	3425.50a	3404.00a	3542.75a	2351.75a	2395.25a	2493.50a	2405.75a	2396.50a	2507.50a
T_7	2046.00f	2008.25g	2214.25g	2125.50g	2112.50g	2242.25f	1589.00g	1623.00g	1747.00g	1690.75g	1652.00g	1777.50g
T_8	1012.50g	1105.50h	1308.50h	1203.50h	1206.25h	1334.75g	1051.50h	1093.00h	1187.25h	1157.00h	1151.00h	1238.75h
LSD at 0.05	11.09	11.02	11.72	14.62	10.70	10.82	5.35	4.77	21.64	6.3	4.97	8.63

Enzyme	**Days of observation after treatment**																	
	Peroxidase (PO)						**Poly phenol oxidase (PPO)**						**Catalase (CAT)**					
Days	**0**	**10**	**20**	**30**	**40**	**50**	**0**	**10**	**20**	**30**	**40**	**50**	**0**	**10**	**20**	**30**	**40**	**50**
T_1	1.79d	2.10de	4.33bc	3.64b	2.80b	4.03c	2.30c	3.24b	4.84a	4.10a	4.14a	5.32b	0.39d	0.54c	0.74d	0.64d	0.59d	0.82d
T_2	1.34e	2.14de	3.69d	3.11d	2.84b	3.78d	2.44b	3.16c	4.43b	3.80b	3.71c	5.07c	0.21f	0.35d	0.54ef	0.42e	0.39e	0.61f
T_3	1.92c	2.27cd	4.15c	3.30c	3.10b	4.36b	2.15d	2.76d	3.80c	3.17c	3.11d	4.16e	0.65b	0.80b	1.28b	0.94b	0.84b	2.18b
T_4	2.16b	3.00a	4.17c	4.11a	4.02a	4.95a	1.72f	2.44e	3.17e	2.71d	3.12d	3.63f	0.59c	0.72b	0.88c	0.80c	0.76c	0.93c
T_5	1.75d	1.96e	4.15c	2.97d	3.89a	4.91a	1.94e	2.30f	3.39d	2.76d	2.71e	4.43d	0.34e	0.45cd	0.66de	0.56d	0.57d	0.75de
T_6	2.39a	2.54b	4.38b	3.33c	3.10b	4.53b	2.71a	3.33a	4.84a	4.11a	4.00b	5.53a	0.91a	1.65a	3.35a	2.23a	2.15a	4.18a
T_7	1.18f	2.39bc	4.71a	3.11d	3.09b	4.89a	1.66g	2.15g	3.80c	2.76d	2.59f	4.14e	0.23f	0.35d	0.53f	0.39e	0.42e	0.64ef
T_8	0.59g	0.68f	1.95e	1.04e	1.03c	2. 05e	0.83h	1.11h	1.95f	1.74e	1.72g	2.29g	0.07g	0.11e	0.22g	0.16f	0.14f	0.27g
LSD at 0.05	0.10	0.21	0.18	0.17	0.31	0.21	0.05	0.07	0.11	0.47	0.06	0.06	0.05	0.11	0.12	0.11	0.07	0.10

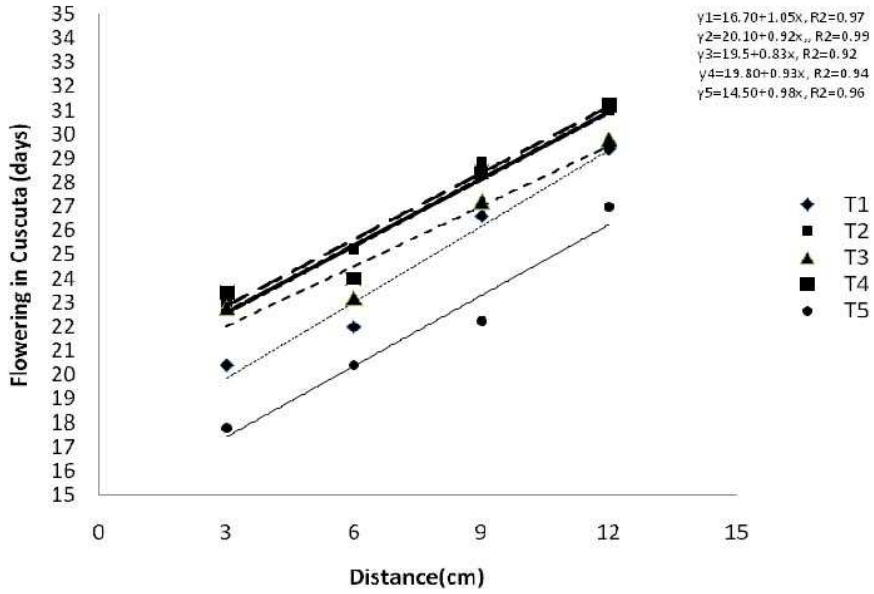

Fig. 1. *Changes to first flowering in Cuscuta in response to different treatments and distance of sowing from the chickpea.*

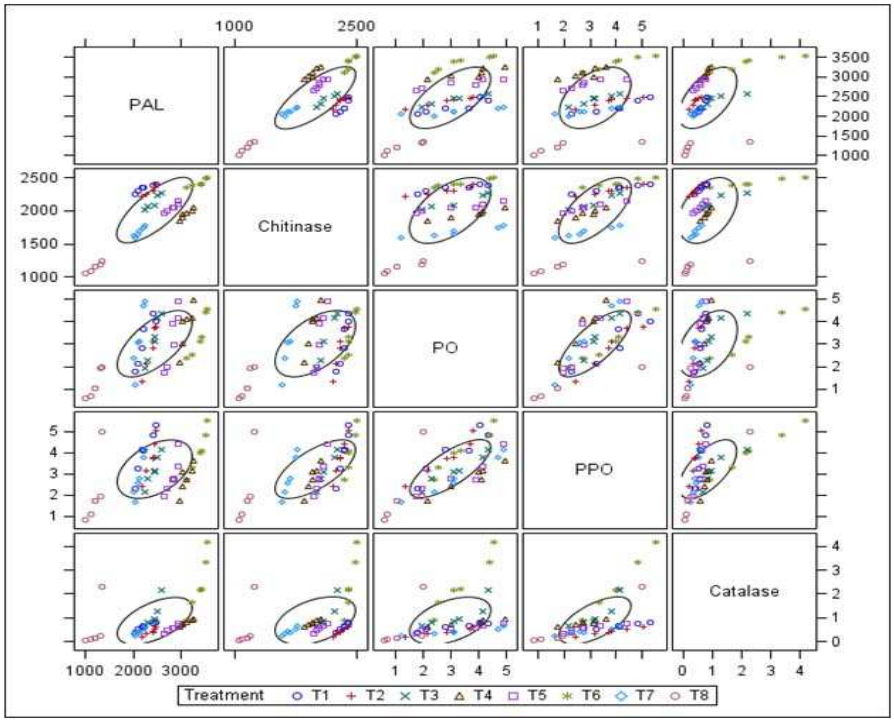

Fig. 2. *Scatter plot matrix showing the relationship among different defense enzymes.*

when sown at 3cm away from the host. This was followed by the treatment with bion (24 days and 25.83% over control).

Linear model was best fitted to the flowering in *Cuscuta* at different distance from host plant chickpea. Results shows that initially, maximum delay in flowering occurs in treatment T_2 (at 20.10 days) followed by treatment T_4 (19.80 days) with slope 0.92 and 0.93 respectively. As the distance of the *Cuscuta* from the host plant increases, delays in flowering in *Cuscuta* also increases linearly.

Understanding the process of their parasitization and development would lead to develop efficient strategies for their management (Westwood *et al.*, 2012). Contrast to the earlier reports about physical and physiological dormancy of *C. campestris* and about a high percentage of newly matured seeds of *C. campestris* not imbibing water to germinate readily (Hutchison and Ashton, 1980) and the need for acid scarification (Jayasuriya *et al.*, 2008), our studies have proved that fresh seeds, before drying in the plants germinates immediately without any need for scarification. This indicates that when sprinkler irrigation is given just before the harvest of the crop,

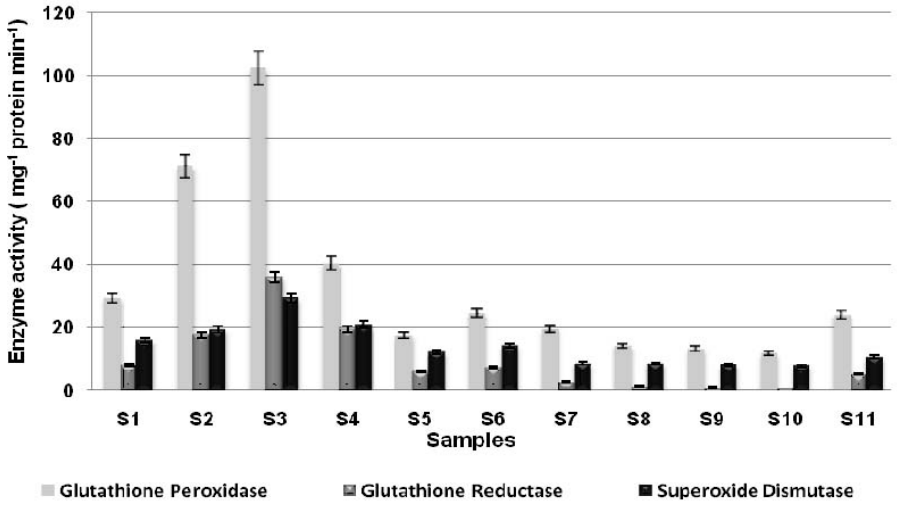

Fig. 3. *Antioxidant enzyme activity in Chickpea and Cuscuta upon treatments with the bioagents and elicitors. Sample details- S_1: T. viride treated hickpea leaves; S_2: P. fluorescence treated chickpea leaves; S_3: 1ppm thiobendazole (Bion @50% a.i.) treated chickpea leaves;S_4: salicylic acid treated chickpea leaves; S_5: Cuscuta infected chickpea leavs (Negative control); S_6: Control (only chickpea leaves);S_7: Only Cuscuta;S_8: Cuscuta from T. viride treated chickpea plant;S_9: Cuscuta from P. fluorescence treated chickpea plant;S_{10}: Cuscuta from thiobendazole (1ppm Bion @50% a.i.) treated chickpea plant; S_{11}: Cuscuta from salicylic acid treated chickpea plant.*

the matured seeds will be germinated and killed during subsequent harvest of the crop. Further irrigation prior to sowing the main crop, to optimum wetness would also result in the suicidal germination of the seeds of *C. campestris*. Further manual cleaning of the twines before they mature would result in the depletion of the parasitic weed seed bank in the soil.

Upon germination, green to yellow fine threads of *C. campestris* grew randomly for a day or and on reaching chickpea, the twines coils around the aerial parts, mainly the stem and leaves, produce haustoria to penetrate the host tissue and vascular system to draw the nutrients and water. Delayed flowering as an effect of bioagents and synthetic elicitor seed treatment could be due to the release of volatiles by the host to deter/ suppress the development of *C. campestris.* It is well established that *T. viride* and *P. fluorescens* application results in the overall development of systemic resistance in the host plants (ISR) (Van Loon *et al.*, 1998).

Systemic resistance induced by antagonists in chickpea: Observation on the effect of the treatments of elicitors on *C. campestris* and chickpea indicated that seed treatment followed by foliar sprays at 20 and 40 DAS was found to have positive effect on the growth and health of the plants.

Estimation of defense enzymes at an interval of 10 days for 50 days indicated the initial increase, reaching a peak and the decline of enzymes activity in the plants (Table 3). This trend shows that the induction is purely temporary and the induction potential of the microbes and the elicitors decreases after a certain period of time (Kannan and Jose, 2009). Repeated application of the bioagents or the elicitors could maintain an enhanced activity of the enzymes which is evident from the fact that the seeds treated plants, followed by foliar spray of the elicitors (treatments T_4 to T_6) had overall more activity of the enzyme when compared with the plants with only seed treatment (T_1 to T_3). Further the bioagents vary in their ability to induce different enzymes viz., *P. fluorescens* was very effective in inducing all the enzymes except CH, while *T. viride* was found to induce more of CH. However salicylic acid was most effective in inducing the enzymes than the microbes. These enzymes are key components of local and induced systemic resistance (Jankiewicz and Kołtonowicz, 2012). Though initially salicylic acid was better than microbes in inducing the defense enzymes, under natural conditions over a longer period of time the antagonistic microbes would build up their populations and induce the plants to produce more of the enzymes, which will not be the case with salicylic acid. Though BTH, a functional analogue of SA, has been reported as a successful resistance activator of plants (Oostendorp *et al.*, 2001) in the current study it was found to suppress the initial growth of chickpea even at a very minimal dose.

The scatter plot matrix (Fig. 2) shows the relationship among five enzymes taken two at a time. Matrices reveal information like clusters and any outlier treatment among many treatments present in the data. In this plot, adjacent plots share common axis. It shows the eclipses which cover the maximum data points in it for different treatments. Those treatment values falling outside the eclipse shows significant difference with other treatment values. It also shows that in most of the comparisons, T_6 outperforms all the treatments and T_8 (control) have outliers and does not

perform well.

Time *vs* treatment interactions were studied for different enzymes and treatments using proc GLM procedure in SAS to know the significance of treatments on each point of time. Results indicated that the enzyme PAL had the highest activity in treatment T_6 and the activity differed significantly for other treatments also. However, with respect to the enzyme PO, the treatments T_4 to T_7 showed significant variations at different points of time, but never showed a constant trend. Again the treatment T_6 was found to be the best one for the enzymes CH and CT during the entire period of observation. All the enzymes except PPO showed significant interaction between treatment and time. PPO did show some significant changes between treatments at the early period of observations, but at later stages, the differences were non-significant. The activities of the antioxidant enzymes were estimated both in chickpea and *C. campestris* to analyze the effect of the treatments with the elicitors. It was observed GPX, GR and SOD were found to be maximum induced (102.36, 36.02 and 29.39 units mg^{-1} protein min^{-1}, respectively) by the application of bion as compared to control (Fig. 3). It was also observed that the antioxidant enzymes were more active in *C. campestris* (24.08, 5.36 and 10.79 units mg^{-1} protein min^{-1}, respectively) upon application of salicylic acid and the activation was significantly high when compared to treatment with the elicitors. In the case of GPX, *P. fluorescens* induced more activity of the enzyme (71.18 units mg^{-1} protein min^{-1}) followed by salicylic acid (40.35 units mg^{-1} protein min^{-1}), while these two treatments were at par in the case of GR and SOD. The biochemical activation and accumulation of defense enzymes, mainly the reactive oxygen scavengers, help in recovery of plants from the damage caused by the invasion of the parasite (Scandalios, 2005; Nyochembeng *et al.*, 2007).

Conclusion

The above study showed that the fresh seeds of *C. campestris* germinate rapidly in a period of 5 to 6 days. This observation would help in suggesting that irrigation immediately before or after harvest of the crop, would induce the germination of *Cuscuta* seeds in soil and after germination, in the absence of the host would die, akin to the suicidal germination strategy followed for *Orobanche* and *Striga* with the use of germination stimulants. Application of *T. viride* and *P. fluorescens* elicited systemic resistance in chickpea, resulting in the increased production of defense enzymes, have better growth and suppresses growth of *C. campestris*. These microbes have already been established for their effective role in suppression of soil borne plant diseases and nematodes in chickpea. Thus the current study helps in emphasizing the application of these two microbes for better production of chickpea. Thus the overall results obtained in the current study gives a positive trend for the management of this dreaded weed in chickpea using the bioagents, which can be easily integrated with the existing management practices at minimal cost.

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