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In vitro effect of chitosan nanoparticles on wilt disease resistance of chickpea by seedlings root feeding of *Fusarium oxysporum* f. sp. *ciceri*

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Abstract
Chickpea is widely consumed dietary legume contributing 18% of global pulse production. Standing third position after beans and peas, chickpea produces about 12.09 MT annually in the world. Unexpected climatic changes imposed several abiotic and biotic stresses on yield and productivity of chickpea. Among them, *Fusarium oxysporum* f. sp. *ciceris* (Foc) is a major soil born pathogen which causes 10-100% yield losses. In earlier studies chitosan and *Bacillus subtilis* sp were reported by many scientists as bioagents in management of diseases in crops. To overcome wilt problem in chickpea, attempts were made to identify the significant use of chitosan nanoparticles and *Bacillus* sp against wilt pathogen. In present experiment, root dip method demonstrated that chitosan and *Bacillus* sp treated gram seeds when fed with *Fusarium* spore suspension survived more days as compared to the untreated seeds. The seedlings with chitosan nanoparticles at 100 and 150 ppm and CNPs + bioagent at all concentrations survived for 22 days without displaying any symptoms of wilting. While in the case of negative and positive control, wilting started at 15 and 17 days of sowing respectively. Whereas, in the case of bioagent alone treated ones started wilting after 18 DAS. It is revealed that chitosan nanoparticles at 100 and 150 ppm when combined with *Bacillus subtilis* showed a synergistic effect of induced disease resistance in chickpea treatments at the early seedling stage for wilt resistance. Therefore, this is very simple and rapid method for screening induced resistance at early stage which will help for evaluation of bioagents for their use.

Keywords: Chitosan, nanoparticles, *Fusarium*, chickpea, root dip method, induced disease resistance

Introduction
Now a days chickpea production and productivity are largely limited due to various abiotic and biotic stresses. Fungal and viral diseases form a major part of biotic constraints in chickpea producing countries (Navas Cortes *et al*., 2008) [1]. Among them, *Fusarium oxysporum* f. sp. *ciceri* causing wilt disease, is a widely spread soil and seed borne pathogen which mainly attacks xylem vessels. As a result, plant dies reducing the yields of crop up to 90 per cent depending on the environment and infected stage of crop (Jendoubi *et al*., 2017) [3].

Fusarium is an anamorph genus with worldwide distribution and a has wide degree of diversity and has been found an important pathogen on a very wide host range of agricultural crops. *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *ciceri* is a serious problem for many plants, and is a troublesome and often serious disease in both dryland and irrigated areas (Kraft, 2000). *Fusarium* spores can survive in crop residues for about 72 months and in soil for more than 6 years, therefore this disease is difficult to control (Haware and Nene, 1984; Singh *et al*., 2014) [12, 5]. Moreover, all high yielding varieties developed so far are susceptible to wilt disease. So, farmers started using unrestricted use of pesticides which accelerated the emergence of resistant strains of pathogens (Levy, 2002; Biyela *et al*., 2004) [6, 7]. Therefore, development of resistant varieties is the best viable method to overcome this disease but unfortunately, the process involved in development and maintenance of uniform sick plots hinders the process (Jimenez-Gasco *et al*., 2001) [8].

With the introduction of Genetically Modified (GM) crops, the application of agrochemicals has reduced significantly (Dunwell, 2011) [9]. GM crops have induced serious concerns about unpredicted change in products of transgenes. Hence, transgenic plants are not adequately accepted (Frewer *et al*., 2013) [10]. So, it is assumed that systemic acquired resistance is the only long-lasting defence mechanism, involving several defence components representing one of the best alternatives besides chemical pesticides, GM crops and biocontrol approaches. Therefore, nowadays non-host resistance is being induced by external application of

compounds or elicitors such as antagonistic microbes, chitosan which could be a breakthrough in plant disease management (Sathiyabama and Manikandan, 2016) [11].
Root submersion method has been widely adopted for screening disease resistance in many crops. Further, use of antagonistic bacteria for management of disease resistance and use of chitosan for enhancing the antimicrobial secretions of beneficial microbes as well as enhancing disease resistance of plants has also been widely explored by various researchers.

Material and Methods

Seeds of chickpea variety Vishwas was obtained from All India Coordinated Pulses Improvement Project, MPKV, Rahuri were used in this experiment to determine the efficiency of chitosan nanoparticles and *Bacillus subtilis* synergistic effect. Seeds were surface sterilized with 1% sodium hypochlorite for 10 min and thoroughly washed with sterile distilled water thrice and used for the experiment.
Chitosan nanoparticles were procured from Vasantdada Sugar Institute, Pune which was diluted appropriately to obtain desired concentration of chitosan nanoparticles. The *Bacillus subtilis* was obtained from Department of Plant Pathology and Agricultural Microbiology, PGI, MPKV, Rahuri (MH). 5 ml of bacterial suspension was added in the test tube and the total volume of solution in test tube was adjusted to 50 ml.

Preparation of *F. oxysporum* f. sp. *ciceri* spore suspension

Inoculum was prepared by making 120 ml potato dextrose media in 250 ml volumetric flasks and autoclaved. A fungal bit of 5 mm^2 diameter *F. oxysporum* f. sp. *ciceri* from an actively growing 7-days old culture were aseptically inoculated in flasks. Then after flasks were placed on shaker and vigorously shaken for 2-3 days at 25 °C. Incubating the culture with shaking enables the uniform distribution of conidia in flask which or else produces only on the surface of the liquid media. Spore suspensions were then filtered and conidia were counted by haemocytometer. Optimum of 5×10^4 conidium per ml were obtained for our study.

Bacillus subtilis and chitosan Inoculum preparation, inoculation and incubation

Seeds were treated with below said treatments of chitosan nanoparticle for overnight. Seeds treated with sterile distilled water served as control. These treated seeds were allowed to incubate in water for about 24 - 48 hrs. Uniformly germinated seeds of various treatments were planted in pot trays (8 cm diameter) filled with sterilized coco peat moss and allowed to grow for one week.
The 7-days old seedlings grown in plot were uprooted, gently washed with distilled water and transferred to tubes with a fungal culture and 15 ml solution in sterile conditions. Isolates of *Fusarium* spp. along with seeds the chickpea cultivar Vishwas was cultivated in glass tubes of 16 mm diameter. Seeds were inoculated with the pathogen (only), pathogen with antagonistic bacterial culture of *Bacillus*, different concentrations of chitosan with pathogen and different concentrations of chitosan with antagonistic bacterium *Bacillus* sp.
Vials were covered with aluminium foil and placed for incubation in glass house for 21 days at a 25 °C day/night temperature with a 12 hour photoperiod. Three replications for each treatment were followed and experiment was repeated twice to obtain accurate results. The experiment with following treatment combination was conducted and observations were recorded regularly for wilt disease incidence.

In vitro Treatment Details

T1 - *Bacillus subtilis* @ 5g/ kg seed
T2 - Chitosan NPs @ 50ppm
T3 - Chitosan NPs @ 100ppm
T4 - Chitosan NPs @ 150ppm
T5 - *Bacillus subtilis* + Chitosan NPs @ 50ppm
T6 - *Bacillus subtilis* + Chitosan NPs @ 100ppm
T7 - *Bacillus subtilis* + Chitosan NPs @ 150ppm
T8 – Negative Control (Inoculum + without bio agent)
T9 - Positive Control (sterile distilled water)

Results and Discussion

This experiment demonstrated that, non-treated plants but root inoculated with *Fusarium* spore suspension causes poor development in root and shoot as compared to CNP and CNP Bacillus treated ones (Fig 1).
Moreover, symptoms were prominent displaying necrosis of roots and death of seedlings in 15 days (Fig 2). In treatments of CNP@ 50 ppm, wilting and necrosis occurred earlier as compared to CNP treatments of higher concentrations and combined treatments of CNP and bioagent. Whereas in positive control (i.e., with sterile distilled water), wilting appeared after 17 days of sowing and in only with bioagent incorporated ones after 18 DAS (Fig 3). On the other hand, treatments of CNPs at 100 and 150 ppm and CNPs + bioagent at all concentrations survived for 22 days without displaying any symptoms of wilting. Therefore, CNPs at 100 and 150 ppm and CNPs when combined with *Bacillus subtilis* was found better to screen the chickpea treatments at early seedling stage for wilt disease resistance.

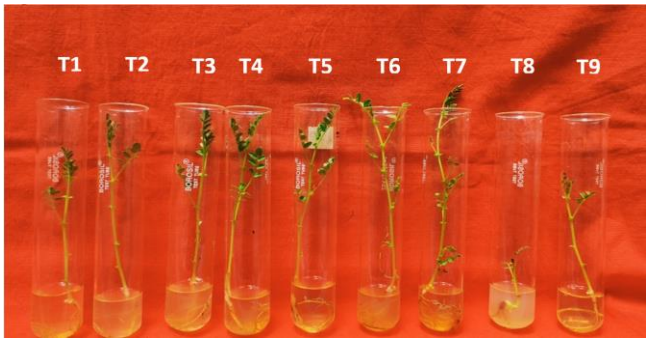

Fig 1: Photograph showing poor development of untreated chickpea seedlings on 9th DAS

Fig 2: Photograph showing symptoms of wilting in untreated chickpea seedlings (T8) on 15th DAS

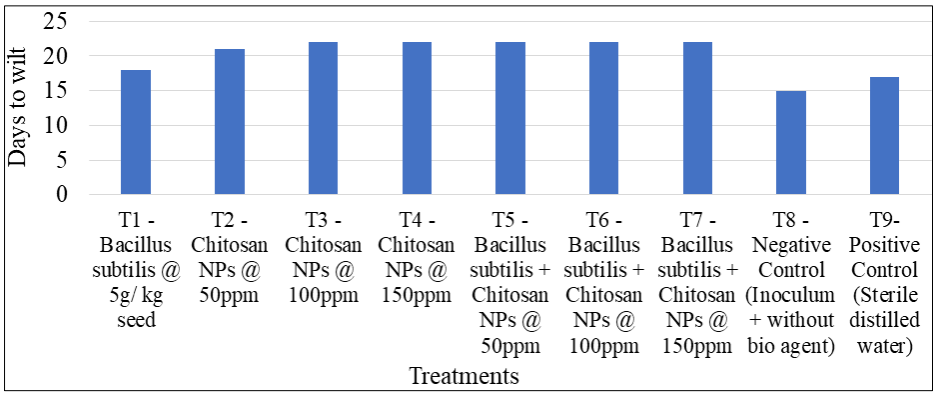

Fig 3: Seedling reaction of treated chickpea seedlings to *Fusarium oxysporum* f. sp. *Cicero*

These results were found to be correlated with the reports of Ravikumar and Babu (2007) [2] where fusaric acid (FA) is root fed to differentiate the resistant and susceptible genotypes. He found that FA less than 25 ppm caused wilting and necrosis early in susceptible ones compared to resistant and at 15 ppm, susceptible ones wilted immediately after 6 days after transplanting compared to resistant ones at 13 DAT.

This *in situ* experiment demonstrated that CNPs at 100 and 150 ppm and with bioagent at all concentrations differentiates the resistant treatments from susceptible ones by causing late wilting in treated seeds. The death due to wilt disease was early by 7 days in non-treated ones as compared to treated ones. Thus, this research proves the combination effect of *bacillus* and chitosan in combating the wilt disease of chickpea. Over all, present study shows that chitosan and *Bacillus* have synergetic effect on controlling wilt disease incidence and were effective over CNPs and *Bacillus* treatments when treated alone.

Conclusion

Here we used a simple technique for evaluating the efficacy of chitosan nanoparticles (CNPs) along with the synergistic effect of CNPs with *Bacillus subtilis.* This technique relies on plant response to toxin when treated with various concentrations of CNPs and bioagents. As a proof of concept, the non-treated seedlings were the most sensitive in the test, whereas the biocontrol agent incorporated seedlings showed resistance to even in the presence of high concentrations pathogen spore mass inoculation. In addition, the bioagents inoculation enhanced shoot growth of the treated seedlings.

These results show the potential of bioagent inoculation for inducing disease resistance for resistance against *Fusarium*. Furthermore, the possibility of using bioagent inoculation as an agent for induced resistance in *Fusarium* was also evaluated by using bioagent as a resistance inducer.

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