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ANTAGONISTIC ACTIVITY OF *RHIZOBIUM* ISOLATES TO FUNGUS *SCLEROTIUM ROLFSII* SACC. CAUSING COLLAR ROT OF SUNFLOWER (*HELIANTHUS ANNUUS* L.).

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ABSTRACT

The study was conducted to isolate the nodule borne *Rhizobium* spp. and to assess their antagonism to *S. rolfsii*, a soil borne pathogenic fungus of sunflower, non-leguminous plant. The bacteria were isolated from cluster bean (*Cyamopsis tetragonoloba (*L.) Taub. plants growing at various locations of Khairpur District, Pakistan. The five strains were found antagonistic on Czapek-Dox agar medium poured dual culture plates to *Sclerotium rolfsii* fungus. Isolate S7 was found highly efficient to check *S. Rolfsii* colony with 21mm inhibition zone followed by S6 (18mm), S9 (17 mm), S1 (13 mm) and S8 (12mm). The strains S7 and S6 were highly efficient to check the *S. rolfsii* growth in soil when inoculated in pot soil. The sunflower plant biomass observed with higher frequencies in dual infested soil (*Rhizobium* isolate S7, S6) with *S. Rolfsii* than control treatments. Thus the isolates S7 and S6 were found to be highly antagonist to *S. rolfsii.*

Key-words: Collar rot of sunflower, *Sclerotium rolfsii*, *Helianthus annuus*, *Rhizobium* spp.

INRODUCTION

Sclerotium rolfsii Sacc. is an important pathogen causing soil borne diseases in at least 500 species of the plants (Aycock, 1966). Sunflower collar rot is one of the most important disease in Pakistan and causing 11-12% yield losses in the field (Mizra and Khokhar, 1985; Gulya *et al*., 1997; Yaqub and Shahzad, 2005). *S. rolfsii* is known pathogen of the sunflower collar rot worldwide (Boland and Hall, 1994). Since the chemical fungicides are quick remedy of the disease control, that has been practiced extensively. The fungicides residue in food has provoked several environment, health hazard and pathogen resistance. Therefore the world is looking for organic and safer remedies.

The plant protection strategies based on antagonistic and biological safe organisms are getting much applause and commercially applied as biological control agents (BCA) (Mathre *et al*., 1999). Rhizobia survive and colonize the roots of non-legumes as efficiently as they colonize the roots of their legume host have been proven (Gaur *et al*., 1980; Chabot *et al*., 1996; Yanni *et al*., 1997; Ladha *et al*., 1998). Present study, therefore, is also expedited the effect of Rhizobia isolates on non-leguminous plant. Since the root nodule borne *Rhizobium* spp. are the potential antagonist of the root borne fungi and *S. Rolfsii* as well (Ehteshamul-Haque and Ghaffar, 1993; Deshwal *et al*., 2003; Ganesan *et al*., 2007; Nahar *et al*., 2008; Yaqub and Shahzad, 2011), therefore present study explored the antagonistic potential of *Rhizobium* isolates from cluster bean, *Cyamopsis tetragonoloba (*L.) Taub., nodules that are reported for stress tolerant bio-control agents (Dhull and Gera, 2017) for antagonism assessment to *S. rolfsii*

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MATERIALS AND METHODS

Isolation of Bacteria: Cluster bean plants were uprooted thoroughly with whole root system from five different agricultural land of district Khairpur. The plant root systems were covered with polyethylene bags separately and transported to the laboratory within 02 hours. The nodules of each collection were surface sterilized with 03% Sodium Hypochlorite (*v/v*) and rinsed five times with sterile distilled water (SDW). The surface sterilized nodule was crushed on a sterilized slide. The crushed/squeezed inner mass of nodule was diluted in SDW in test tube and streaked on yeast extract Mannitol agar (YEMA) amended with 10 ppm of Congo red dye, as describe by

Somasegaran and Hoben (2012). The manifested bacterial colonies were transferred aseptically to YEMA slants and stored at 04ºC for 30 days.

Confirmation test: The *Rhizobium* isolates were confirmed with the nodulation test through artificial inoculation of bacteria in sterile soil and sown with mung bean, *Vigna radiate* (L.) R. Wilczek. The nodulations on the roots of the test plant confirmed the *Rhizobium* spp. of the isolates.

The live fungal culture of *S. Rolfsii* was obtained from Department of Agriculture and Agribusiness Management, University of Karachi, on Potato Sucrose Agar (PSA) medium poured slants. The culture was multiplied on PSA media for hyphae and sclerotia production at 28 ºC inside the growth chamber.

***In vitro* dual culture antagonistic test:** The antagonistic activity of the bacteria to *S. Rolfsii* was studied on Czapek Dox agar (CDA). The *Rhizobium* isolate was streaked on one margin of the culture (09 cm) Petri plate and the fungal fresh mycelia (09mm fresh culture disk) was placed at opposite margin. The control plates were inoculated with the fungi and opposite area and streaked with sterile water. While the bacterial control plates were streaked with bacteria on one side. The dual cultures and control plates were placed at 28 ºC for seven days and calculated the growth of the fungal and bacterial colony diameters (T) as compared to control (C).The zone of inhibition between the fungi and bacteria culture was also measured. The percent inhibition (I) was calculated by the formula I=100(C-T)/C, The experiment was replicated five and repeated two times.

***In vivo* screen house experiments:** The pot experiments were conducted to study the pathogenicity and antagonistic effect of *S. rolfsii* and *Rhizobium* isolates on host sunflower plants grown in silty-loam+5% farm yard manure (FYM) soil. The soil was sterilized in autoclave at 120 ºC /15 lbs. and cooled. The soil filled into 18x30 cm pots aseptically and inoculated with 03 cm *S. Rolfsii* hyphae disk and moistened with SDW.

The treated soil was kept in pots for 12 h. in growth chamber. The sunflower seeds were surface sterilized (ST) with 2% sodium hypochlorite water solution and five times rinsed with SDW. The 01% sterilized agar water solution was prepared and inoculated with *Rhizobia* isolates. The solution was maintained with $1.0x10^8$ cfu/mL of the bacterial culture through dilution formula using haemocytometer and each pot irrigated with 01 ml of the bacterial inoculums. The ST seeds were placed in the bacterial solution for one hour and sown in the treated soil of the pots. The positive control pots and their seeds were not inoculated with the pathogen and *Rhizobium* spp. Isolates; other negative control pots were sown with ST seeds free of bacteria.

Each pot was sown with three seeds, when the seedling developed healthy amongst three, single seedling was left to grow. The pots were regularly irrigated with SDW. The root-shoot length, root and shoot weight were measured, of the sunflower seedling, after 20 days. For the pot experiment the treatments were used as T1= positive control soil with no addition of bacteria and fungi, T2= negative control soil added with the fungal mycelia only, T3= *S. rolfsii* and isolate S7, T4=*S. rolfsii* and isolate S6, T5= *S. rolfsii* and isolate S9, T6= *S. rolfsii* and isolate S1, T7= *S. rolfsii* and isolate S8.

Statistical analysis: All the experiments were designed according to complete randomised block design model (CRBD), having five replicates, each experiment repeated twice. The data were analysed for Duncan's Multiple Range Test (DMRT) for significant differences in mean values and ANOVA test at $P<0.05$, using IBM-SPSS19 software.

RESULTS AND DISCUSSION

There were ten bacterial isolates from cluster bean nodules. *Rhizobium* spp. nodulation characteristics were studied in the isolates. Among the ten isolates eight were confirmed by nodulation tendency in mung bean seedlings. The temperature of June and July 2017 ranged from 28-47 ºC, which are causing draught conditions that has also direct impact on plants, even the stress were high but the sampled plants were robust growing, thus the tolerance may be plant specific or *Rhizobium* support as reported by (Suárez*et al*., 2008). All the *Rhizobium* isolates are not significant disease antagonists (Ganesan *et al*., 2007), present study also screened three important strains that effectively controlled the fungal growth *in vitro*. It is also well reported that the stress environments nourish stress tolerant *Rhizobium* spp. (Zahran, 1999); present study also supports the view point that the stress tolerant strains are available in high temperature environments.

The bacterial isolates S7, S6, S9 and S1 were found effective to check the *S. rolfsii* growth. The isolate S7 caused 79.2% growth inhibition and 21mm zone of inhibition to the fungi on dual culture, which is followed by S6, S9 and S8 with 71.2% (18mm), 63.1% (17mm) and 63.1% (17mm), respectively. While other six strains were found

with few to zero inhibition attributes to the fungi (Fig. 1). The bacterial antagonism caused differences in mycelia, sclerotia and colony texture at antagonist experiments (Fig. 2C).

Table. 1. The nodules collection information of cluster bean is given with geographical location, collection dates and confirmations of bacterial isolates as *Rhizobium* spp. *in vivo*.

Isolates	Location	Collection Dates	Nodulation Test
S1	27°30'02.51"N 68°26'12.39"E	15-06-2017	+ve
S2	27º18'36.93"N 68º35'18.84"E	22-06-2017	-ve
S3	27º08'25.22"N 68º29'03.87"E	30-06-2017	+ve
S4	27º08'22.75"N 68º29'04.93"E	8-07-2017	+ve
S5	27º29'59.60"N 68º25'56.48"E	12-07-2017	+ve
S6	27º30'01.97"N 68º26'11.84"E	12-07-2017	+ve
S7	27º30'00.60"N 68º26'00.29"E	12-07-2017	+ve
S8	27º29'57.06"N 68º25'59.49"E	28-07-2017	+ve
S9	27º31'03.39"N 68º25'48.42"E	28-07-2017	+ve
S10	27º18'52.70"N 68º36'36.84"E	28-07-2017	-ve

+ve refers to the formation of nodule and –ve to those isolate couldn't form nodules and corresponded as non *Rhizobium* isolates.

The present study confirms different reports for the antagonistic activity of *Rhizobium* spp. against *S. Rolfsii* (Manasa *et al*., 2017), the study also reveals cluster bean nodules borne *Rhizobium* isolates have not yet been screened for disease antagonism, although the cluster bean nodules are highly stress tolerant (Dhull and Gera, 2017). Manasa *et al*., (2017)reported 30 to 50% inhibition against *S. Rolfsii* by *Rhizobium* isolates of leguminous origin. Ganesan *et al*., (2007) found that the *Rhizobium* strains were able to restrict *S. Rolfsii* growth from 6-60% on dual culture plates.

Present study also confirms and reports more efficient inhibition of the *S. Rolfsii* by *Rhizobium* isolates, the reason behind the higher efficiency might be origin of the isolates and their presence in hot stress sustaining host plant i.e. *C. tetragonoloba*, the cluster bean.

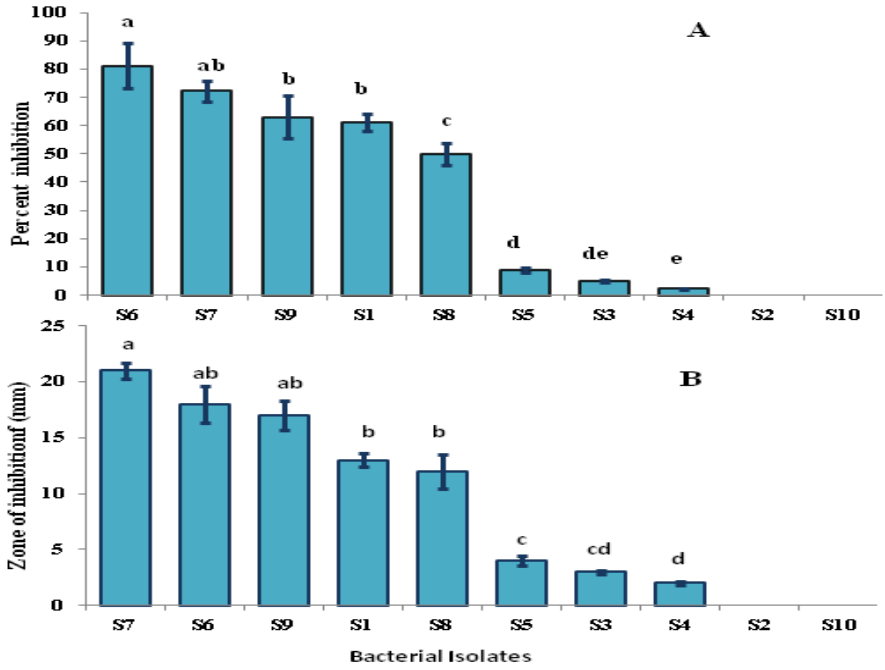

Fig. 1. Inhibition percent of *S. rolfsii* isolates (A) and zone of inhibition to the *S. rolfsii* (B) by *Rhizobium* spp. at dual culture antagonism experiments. The graph bars with same alphabetical annotations are not significantly different in the group according to Duncan's Multiple Range Test (DMRT) at $P<0.05$.

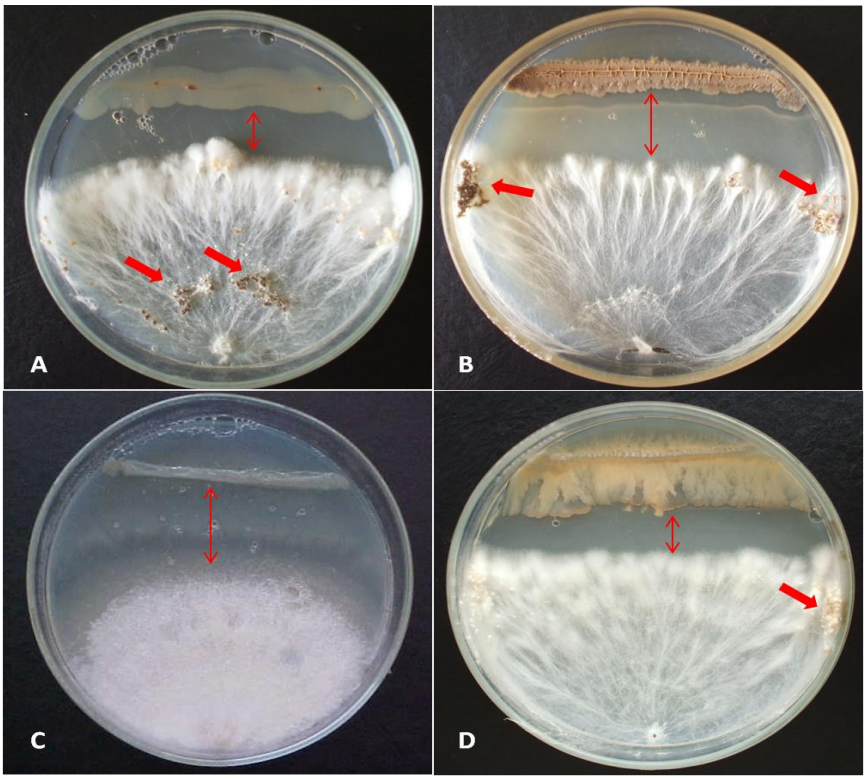

Fig. 2. Dual culture experiments showing the inhibitions of *S. rolfsii* by *Rhizobium* isolates, whereas the isolate S1 mildly inhibited the growth of the fungus, having a good number of sclerotia formation and mycelia mats (**A**). The isolate S6 shows the restriction of fungal growth at distance with small size sclerotia of the fungi (**B**). The isolate S7 exhibits the higher tendency of restriction in mycelia growth, mate formation and development of scrlerotia (**C**). The isolate S9 shows lower restriction in the fungal growth (**D**). The arrow marks refer to the sclerotia formation (single head arrow) and the zone of inhibition (double head arrow).

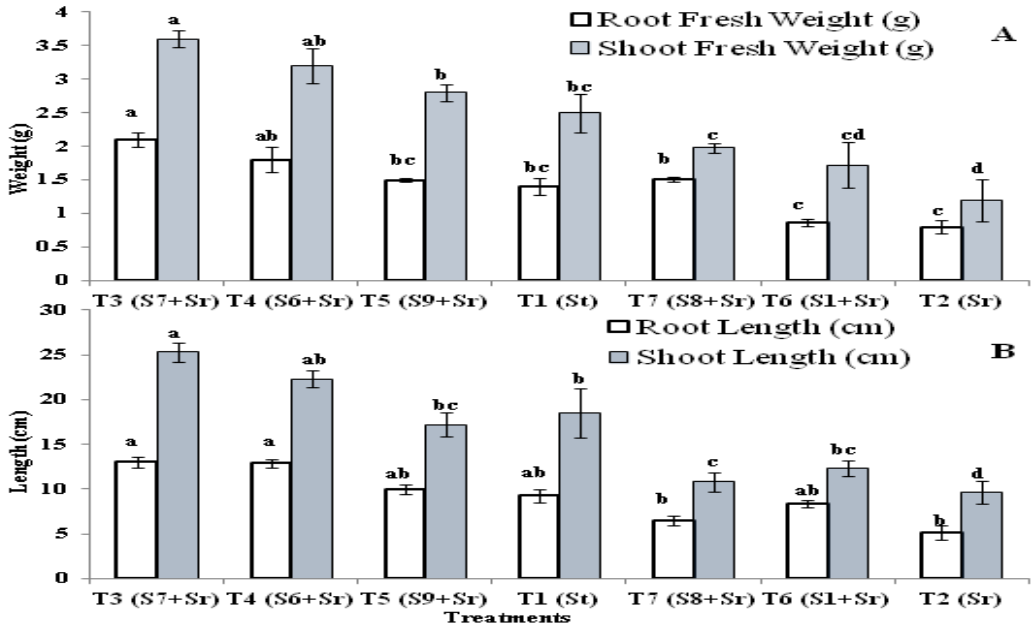

Fig. 3. Antagonistic effects of Bacterial inoculums on sunflower saplings grown in soil with different treatments in terms of Plant fresh weight (**A**) and Plant height (**B**), whereas (**st**) Sterilized soil, (**Sr**) *S. rolfsii*, (**S**) *Rhizobium* spp. strain, (**St**) sterilized soil and Treatment numbers are abbreviated as (**T**). The graph bars with same alphabetical annotations are not significantly different in the group according to Duncan's Multiple Range Test (DMRT) at $P<0.05$.

In screen house conditions, *S. rolfsii* restricted the growth and caused the symptoms of collar rot on sunflower sapling in pots after 30 days. Meanwhile, the *S. rolfsii* has not caused disease severity in sunflower plants, where it has been co-inoculated with *Rhizobium* isolates in the pot soil (Fig. 3). The *S. rolfsii* eventually was suppressed in the terms of plant growth by *Rhizobium* isolates. The *Rhizobium* strain S7 exhibited the highest plant growth in dual inoculated soil as compare to control treatment with the fungi in the soil, the plants grown with 5.7 g biomass, which is the highest and followed by 5.0g (S6) and 4.3g (S9). The sterilized soil was found more effective than S8 and S1 isolates dual treatments. This trend shows decrease in inhibition efficiency of *Rhizobium* isolates *in vivo* (Fig. 1). While the *Rhizobium* strains S7, S6 and S9 have potential to suppress the plant pathogenic fungi in soil as reported earlier for various *Rhizobium* strains (Antoun *et al*., 1998).

This is highly reported that the *Rhizobium* strains are very good bio-control agents to control soil borne plant diseases (Ehteshamul-Haque and Ghaffar, 1993; Deshwal *et al*., 2003; Ganesan *et al*., 2007). Present study suggests the protective effect of *Rhizobium* in soil amendment and it has potential to inhibit soil borne fungi *S. Rolfsii* in soil.

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