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Effect of pH on stability, Sunflower growth promotion and biocontrol potential of a talc-based formulation of *Pseudomonas fluorescens* UTPF61

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Abstract

Sunflower is attacked by many phytopathogens which one of the most important of them is *Sclerotinia sclerotiorum*. Application of *Pseudomonas fluorescens* UTPF61, as an antagonistic bacterium, is one of the successful ways to control this disease. Also different chemical factors in natural conditions cause variation in antagonistic activity of strains. One of them is pH factor which has an important role in the efficiency and survival of biocontrol agent. The aim of this study was to find the efficient pH that increases the efficiency and long stability of UTPF61. This pH data is also useful in economic purposes, especially in industrial and half-industrial scale of bacterial productions. Formulations are one of these functional products. Bacterial existence in Talc-based formulation was studied after growing up in media with pH stresses in between 4-10. This study was carried out under *in vivo* conditions. The formulations were stored at 90 consecutive days and two different temperatures, room temp (26 ºC) and refrigerator temp (4 ºC). The bacterial ability was tested to suppress the development of Sclerotinial damping of sunflower, caused by *S. scleotiorum*. Formulation prepared with bacteria, which had grown up in pH7 and stored in 4 ºC, had the longest stability and biocontrol activity, also this formulation showed significant effect on sunflower growth compare to control (PGPR effect). So, on the basis of the all out coming results, it seems that medium with exact pH7.0 showed appropriate ability to use in commercial production of bacterial agent, also had maximum growth yield of sunflower in lab and green house conditions.

Keywords: Antagonistic bacteria; Formulation; pH; Sclerotinial damping off; Sunflower.
Abbreviations: *P.- Pseudomonas*; *S.- Sclerotinia*; pH- Power of hydrogen (concentration of hydrogen, is a measure of the acidity or basicity of an aqueous solution. Pure water is said to be neutral, with a pH close to 7.0 at 25°C. Solutions with a pH less than 7 are said to be acidic and solutions with a pH greater than 7 are basic or alkaline; PGPR- Plant Growth Promoting Rhizobacteria.

Introduction

Sunflower (*Helianthus annus* L.) is vulnerable to some pathogenic agents, especially the fungus *Sclerotinia sclerotiorum* deBary which is a major soilborne plant pathogen (Agrios, 1997). This fungus infects nearly 400 plant species from 75 families and causes economic damage to a wide range of crops (Boland and Hall, 1994). The most obvious sign of *S. sclerotiorum* is the appearance of white fluffy mycelial growth that will later produce sclerotia. It is responsible for causing root, crown and stem rots. It has a world-wide distribution; however it is most prevalent in cool moist regions. To date, no known field-resistant cultivars are available to this disease*;* also, there are no chemicals registered for 100% control of wilt or ascospore infections of sunflower (Steadman, 1979; Expert and Digat, 1995). Hence, once the pathogen is established in the field, it remains indefinitely because susceptible crops of sunflower will increase the population of the pathogen. Particular bacteria in certain natural environments prevent infectious diseases of plant roots. Artificially application of antagonistic bacteria is a successful way to control this kind of diseases (Weller, 1988; Haas and Defago, 2005). A very notably group of biocontrol PGPRs is fluorescent pseudomonads that protect a range of crop plants from important, mostly fungal, root pathogens (Defago and Hass, 1990; Expert and Digat, 1995; Haas and Defago, 2005). One of these Pseudomonads was *P. fluorescens* UTPF61 which had been selected on the basis of growth promotion activity on sunflower, production of HCN and protease (Heidary, 2008). Bacterial formulation is a product that consists of bacterial cells and a kind of preservative which use to control soilborne diseases. The bacterial component of formulation needs to survive for a considerable length of time and to have *in vivo* efficacy (Bora, 2004). The shelf-life of environmentally sensitive micro-organisms continues to be a challenging and success-limiting step in development of biocontrol products (Paau, 1998). While fluorescent pseudomonads have significant potential as biocontrol agents, they are very sensitive to environmental factors, in particular temperature and acidity (O'callaghan *et al.*, 2006). Also different physical and chemical factors in natural conditions cause variation in antagonistic activity of strains (Flahaut *et al.*, 1997). Pretreatment a regular rhizospheric microbe with a moderated chemical stress will turn it into a PGPR which will be very useful for biological control of soil diseases. pH factor of culture medium has an important role in the efficiency and survival of biocontrol agent. Preset the bacteria with mild stresses like temperature, acidity, starvation and humidity will result in bacterial resistance against more powerful and effective stresses. In many cases these mild stresses cause cross protection in these bacteria (Flahaut *et al.*, 1997). Methylglyoxal which is a chemical plant product accumulate under various stressful conditions. The stress-induced increase in methylglyoxal level has an important role in conferring tolerance to plants under stress

conditions. Every single test condition factors are very important. If one of them changes, the whole test would be influenced. So presenting the exact optimum pH for each bacteria is very significant. Also the objective of this research was to test the effect of pH on performance of *P. fluorescens* UTPF61 bioformulation under lab and green house conditions.

Results

Survival of P. fluorescens UTPF61 in talc-based formulations

The population of *P. flourescens* UTPF61, which grown in each pH, was measured right after preparing the formulations (Fig.1). The initial high populations of UTPF61 in the formulations were not necessarily sustained during the storage at 4°C and 26°C. There was a subsequent decline in the population because of drying stage; 30 days after incubation the bacterial population decreased 10^2 cfu/g in talc-based formulations from the primary population (Fig.2,3). In the experiment measuring long-term survival of UTPF61 strain, the formulation of this strain that grown in pH7 was found to be better, yielding populations after 90 days of storage (Fig. 4).

Efficacy of the formulations in the 90 days trial on sunflower growth (PGPR activity)

The growth ratings were recorded in the treatments in 20th day after sowing. The results are given on figures 5 to 10 as explained below. For the formulation that stored at 26°C, wet weight of sunflower root and stem are shown in Fig. 5 and dry weight of sunflower root and stem in Fig. 6. The most weight of wet root and stem for the formulation stored at 26°C, related to the bacteria which had grown in pH7 and showed a significant difference compare with control. The least weight belonged to the bacteria had grown in pH6. For the formulation stored at 4°C, wet weight of sunflower root and stem are shown in Fig. 7 and dry weight of sunflower root and stem in Fig. 8. In this temperature also the most weight of wet root and stem related to the bacteria had grown in pH7 and showed significantly difference compare with control. The least weight belonged to the bacteria had grown in pH10. Comparison of the formulations stored at 4°C and 26°C showed the formulation which stored at 4°C had the most influence on the sunflower growth after the storage period (Fig. 9, 10).

Efficacy of the formulations in the 90 days trial on biocontrol ability

This bacterium produces an antifungal substance that promotes lysis of fungi mycelium and inhibition of ascospore germination (Andreoli *et al.,* 1993). In the presence of *P. fluorescens* strains on sunflower plants, challenging with the ascospores of *S. sclerotiorum*, the bacteria motivated the plant to increase levels of hydrolytic enzymes including chitinase and b-1,3-glucanase. In addition, it also triggered the expression of the pathogenesis-related protein PR3. Field studies indicated that the rate of disease control achieved by *P. fluorescens* was comparable to the rate obtained by the fungicide Rovral Flos (iprodione) (Fernando *et al.,* 2007). In total, suppression of *Sclerotinia* stem rot of sunflower by Pseudomonas strains depend upon LP (lipopeptide)

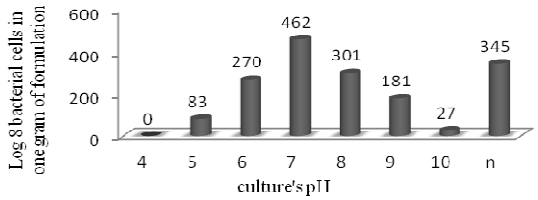

Fig 1. First measured population of *P. fluorescens* strain UTPF61 grown in different pHs. Differences between treatments as shown in all the figures were determined by Duncan's Multiple Range Test (DMRT) at 5% significance level.

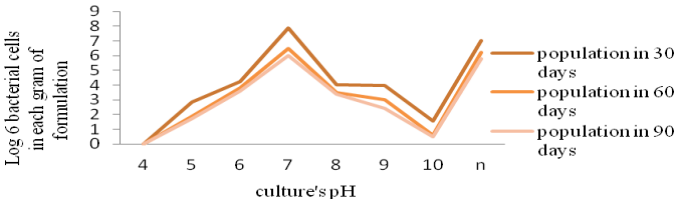

Fig 2. Population of bacterial formulation grown in different pHs after storage at 4°C.

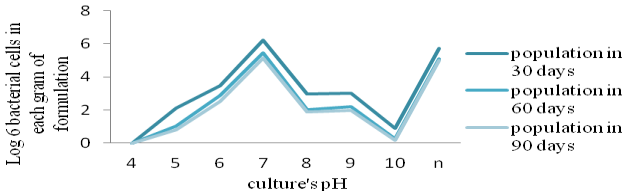

Fig 3. Population of bacterial formulation grown in different pHs after storage at 26°C

production and a functional Gac system (Berry, 2010). In the green house trials, seed-bacterization by talc-based formulation which stored at 26°C and pH7, significantly reduced the percentage of wilt severity caused by *Sclerotinia sclerotiorum* (75%), compared with the control (+) treatment. Control treatment was treated with water in 90 days after sowing the seedlings (Fig. 11). For the formulation stored at 4°C and pH7, it has also shown maximum effect on reducing the wilt of sunflower (Fig. 12). Comparison of formulations stored at 4°C and 26°C showed that the formulation stored at 4°C had the most impact on reducing the percentage of wilt severity caused by *S. sclerotiorum* on sunflower plants (Fig. 13).

Discussion

Various strains of *P. fluorescens* have been found to be effective in plant growth promotion (Raj *et al.*, 2004; Weller and Cook, 1983; Elliott *et al.*, 1984; Miller *et al.*, 1990; Kloepper *et al.*, 1991). These PGPRs inhabit on plant roots and affect plant growth promotion by mechanisms such as increased solubilization and uptake of nutrients and/or production of plant growth regulators (Kloepper *et al.*, 1989; Arshad and Frankenberger, 1991). Therefore, in this research

we directed our efforts to select growth pHs of *P. fluorescens* which were more effective on seed invigoration and improvement of seedling growth. Such studies are considered an efficient way to screen environmental factors for growth of PGPR strains to be more effective (Khalid *et al.*, 2001). As indices, dry and wet weight of seedling studied, and out of 8 pHs tested in this experiment, pH 7 significantly (P=0.05) increased sunflower growth over other pHs and control. So the most consistently performing UTPF61 in seed invigoration and improving plant growth, was grown in pH7. Seed treatment with cell suspensions of *P. fluorescens* has been found to be effective in the control of several diseases (Weller, 1983; Trapero-Casas *et al.*, 1990; Parke *et al.*, 1991). For commercial usage, this methodology would be impractical due to difficulty in handling, transport and storage (Digat, 1990; Ramamoorthy *et al.*, 2001). The commercial use of fluorescent pseudomonads requires inoculum that retains high cell viability and can easily be transported and applied to seed (Kloepper and Schroth, 1981). Fluorescent pseudomonads have been reported to survive in certain dry formulations (Connick, 1988). It has been reported that the populations of fluorescent pseudomonads did not decrease in the talc mixture with 20% xanthane gum after storage for 2 months at 4°C (Kloepper and Schroth, 1981), and in the vermiculite-based dried formulation after storage for 6 months at 4°C (Connick, 1988). In the present study, we have shown that the UTPF61 strain of *P. fluorescens* could survive well in talc-based formulations which stored at 4°C and 26°C for a period of 90 days. Development of dried powder formulation of antagonistic bacteria is especially important for seed-treatment (Ramamoorthy *et al.*, 2001). Kloepper and Schroth (1981) developed a talc-based powder formulation of plant growth-promoting rhizobacteria (PGPR) for inoculation of potato seed pieces. In the present studies, we have shown that the talc-based formulation of *P. fluorescens* strain UTPF61 was found to be more successful than the other treatments because of its efficacy at 90 days after transplanting. In the case of our trial, the seed-bacterization technique was used to introduce the strains of *P. fluorescens* into the system. As a result of our trial, *P. fluorescens* strain UTPF61 protected the sunflower plants at a level of 75% (formulation stored at 26°C) and 100% (formulation stored at 4°C) compared with the treatment of the pathogen alone on day 90th after sowing. When the stability and efficacy of the product was tested under greenhouse conditions, seed-bacterization with the powder formulations could provided better root colonization than aqueous preparations (Kloepper and Schroth, 1981; Vidhyasekaran and Muthamilan, 1995; Ramamoorthy *et al.*, 2001). Seed-bacterization with bioformulation is known to increase the population of *P. fluorescens* more than 10-fold in seed (Vidhyasekaran and Muthamilan, 1995). It is known that fluorescent pseudomonads were the most common population of the rhizosphere layer (Pietr and Kempa, 1987). Moreover, fluorescent pseudomonads have been considered by most researchers as the ideal biocontrol agent of soilborne pathogens (Cook and Baker, 1983). Since many Pseudomonas spp. have a high rhizosphere competence, there should be intense competition for Fe^{3+} at the rhizoplane when the Pseudomonas produces siderophores. Therefore, Fe^{3+} is bound in such a way that is unavailable to *Sclerotinia*. In our studies, formulation of *P. fluorescens* strain UTPF61 reduced Sclerotinial wilt of sunflower by 75% and 100% by 90 days after transplanting. Suppression of the disease for such a long period may not be explained by just the inhibitory effect of siderophores on spore germination. This situation may be

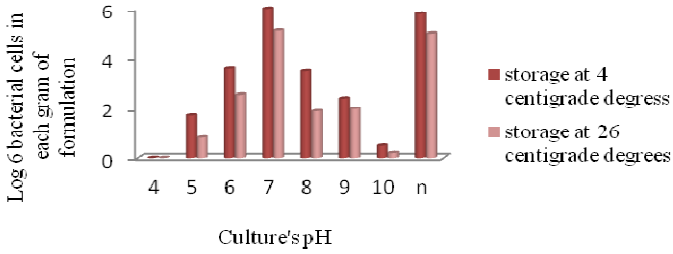

Fig 4. Compare of 90th day population from the formulation stored at 4°C and 26°C.

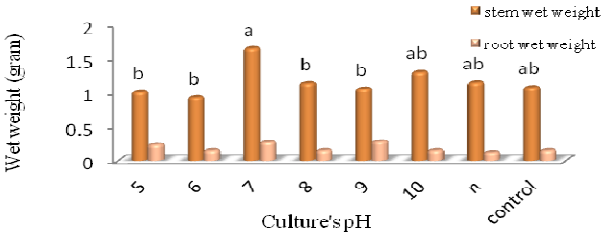

Fig 5. Influence of formulation stored at 26°C on wet weight of sunflower.

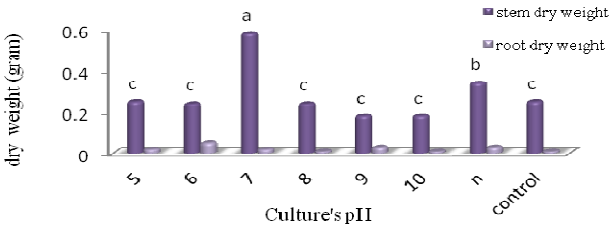

Fig 6. Influence of formulation stored at 26°C on dry weight of sunflower.

explained by the induction of resistance in the host plant by biocontrol agents as another mode of action. That may suppress Sclerotinial wilt, besides microbial competition (Fuchs and Defago, 1990; Van Loon *et al.*, 1998). In addition, siderophores and more specifically pyoverdines have been shown to play a role in the induction of resistance in the host plant against different diseases (Maurhofer *et al.*, 1994). The *P. fluorescens* formulations can be effectively used as seed treatments to control *Sclerotinia sclerotiorum*. Additional studies should be continued to define details of possible mechanism(s) and commercialization methods such as fermentor system.

Materials and Methods

Biocontrol strain

Pseudomonas fluorescens UTPF61 had been selected from 40 strains of fluorescent pseudomonads on the basis of dual culture assays, growth promotion activity on sunflower and production of HCN and protease (Heidary, 2008). This

endophytic strain was isolated from healthy roots of sunflower plants. To diagnose this agent, Laboratory Guide for Identification of Plant Pathogenic Bacteria was used (Schaad, 2001). Stock cultures were prepared for storage at -80°C in 1.5 ml vials by mixing equal volumes of 50% glycerol and 24 h culture broth (from single colony inoculum, 25 ml Luria Bertani medium, 100 ml flask, 130 rpm).

Pathogen

Preparation of fungal isolate

Pure culture of *Sclerotinia sclerotiorum*, was obtained from Dr. Nikkhah, Mycology Lab, Dep. of Plant Pathology, college of Agriculture, Tehran University, Iran. To diagnose this agent in sunflower, Kohn key was used (Kohn, 1979; Prat, 1992; Steadman, 1994; Hanlin, 1998).The fungus was grown at 25°C and maintained on potato dextrose agar (PDA, Difco, 39 g PDA L-1 of distilled H2O, pH = 7.2). All cultures stored at 4°C and sub-cultured as needed. **Pathogencity test:** The pathogenicity of the isolate was established in the beginning of the experiment with the exact conditions that was used in green house trials. Sunflower cv. Azargol seeds were surface-sterilized in 1.5% NaOCl for 5 minutes (Chen and Wu, 1999) and rinsed five times with sterile distilled water, then pre-germinated in 15% water agar for 48 h at 26°C in an incubator. Sandy loam soil with pH 7.5 was disinfected in autoclave and used for each pot. Seeds were sown in soil moistened (3:1, vol/vol). Plastic boxes (11 × 17 cm) were filled with 200 grams of moist soil and 3 seeds were planted in 1-cm-deep holes. One sclerot of *S. sclerotiorum,* approximately 0.7 to 1 cm was added near the sunflower seed (Huang, 1993) and at last 2 cm of sterilized soil were added onto this collection. For the control treatment the seeds were dipped in 0.15% benomyl. The control (+) treatment included only the pathogen. The sunflower seedlings were grown at 22±3°C, 16 h day light and 8 h night for 20–25 days. Seedlings were watered 3 times a week with tap water.

Flask culture studies

All assays in this work were performed using King's B medium, containing in grams per litre: Peptone 20.00, Heptahydrated Magnesium Sulfate 1.50, Potassium Hydrogen Phosphate 1.50 and Bacteriological Agar 15.00 (King *et al*., 1954). This medium was autoclaved for 15 min at 121ºC.

P. fluorescens UTPF61 growth under different pH conditions (preset the bacteria)

pH of King's B liquid cultures were adjusted on 4.0, 5.0, 6.0, 7.0, 8.0 9.0, 10.0 by the addition of sodium hydroxide (NaOH) and hydrogen chloride (HCl). One treatment contains King's B medium without pH adjustment and were kept as normal ph (n=7.2-7.4). All the flasks were autoclaved for 15 minutes in 121°C. The bacteria was grown overnight at 26°C in Nutrient Agar medium and it spectrophotometric adjusted to c. 10^6 CFU ml^{-1} in sterile distilled water. Cell density was measured at 600 nm by Spectrophotometer T70 UV/VIS; thereafter the adjusted population of bacteria was added into 40 ml of King's B medium. Growth media were incubated for 48 h at 130 rpm and 26±1°C in the dark rotary shaker incubator. Each treatment was replicated three times and arranged in a completely randomized design.

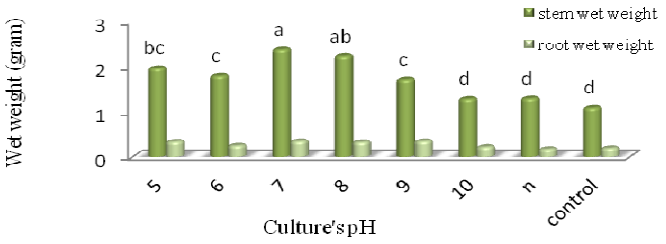

Fig 7. Influence of formulation stored at 4°C on wet weight of sunflower.

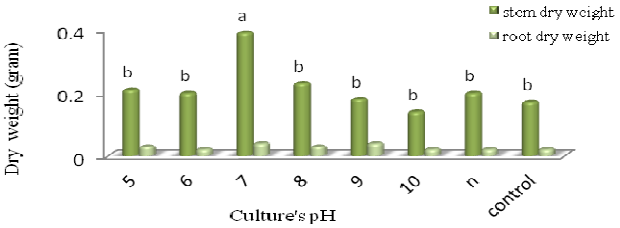

Fig 8. Influence of formulation stored at 4°C on dry weight of sunflower.

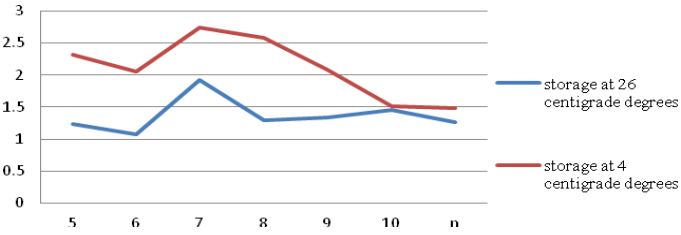

Fig 9. Compare of formulations stored at 26°C and 4°C on wet weight of sunflower.

Separation P. fluorescens UTPF61 from media and preparing the talc-based formulations

Bacterial suspensions were centrifuged for 20 min at 6,520 g. Pellets were resuspended in 0.1 m $MgSO_4$ in a ratio of 1:1 (w/v). Bacterial suspensions in 0.1 m $MgSO_4$ were mixed with a 10% (v/v) glycerol, which was used as a bacterial preservative. This suspension was then mixed with an equal volume of autoclaved 1.5% Na-Alginate (Bashan, 1986; Digat, 1990). The bacteria–Na-Alginate mixture was mixed with sterilized talc at the ratio approximately four times the volume of the bacteria– Na-alginate mixture (1:4, v/v) (Kloepper and Schroth, 1981). The resulting mixture was thinly spread over a glass sheet and air-dried in a laminar air-flow cabinet at 24°C for 1 h to form a slightly moistened powder (15% moisture content). After drying, bacterial formulations were powdered in a blender and stored in glass bottles with lids as small volumes (with 10 grams capacity) (Suslow and Schroth, 1982; Connick, 1988; Petrolini *et al.*, 1998). Bacterial formulations were stored at 4°C and 26 °C for 90 days.

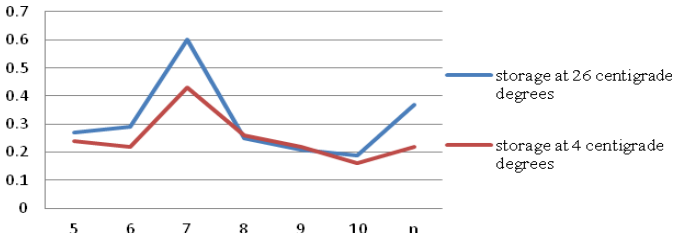

Fig 10. Compare of formulations stored at 26°C and 4°C on dry weight of sunflower.

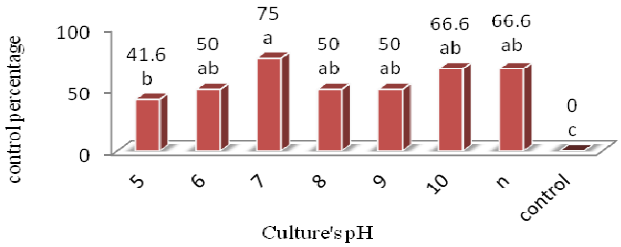

Fig 11. Disease control percentage by formulation stored at 26°C.

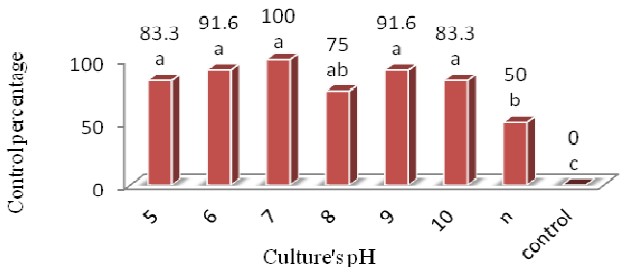

Fig 12. Disease control percentage by formulation stored at 4°C.

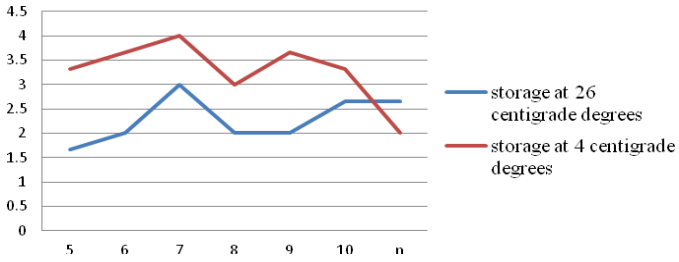

Fig 13. Compare of biocontrol ability between formulations stored at 26°C and 4°C.

Survival of P. fluorescens UTPF61, grown under different pH conditions, in formulations

The antagonistic bacteria in each formulation were monitored *in vitro* with respect to their shelf-life and viability under these storage conditions for this period. Survival of bacterial population in the formulations was assessed at 90 days intervals using King's B medium by dilution plate method (Vidhyasekaran and Muthamilan, 1995). There were three replications per treatment. pH adjusted cultures containing bacteria were used for each test and cultures without pH adjustment were used as control (n = normal).

Green house trials

The talc-based WP (Wettable Powder) formulations of *P. fluorescens* UTPF61, which grown under different pH conditions, were tested for their efficacy to control Sclerotinial damping of sunflower in the green house trials. The bacterial formulations were suspended in distilled water to the end concentration of 10^9 cfu/ml. One seed-lot of sunflower cv. Azargol was used in the experiments. Seeds were surface-sterilized in 1.5% NaOCl for 5 minutes (Chen and Wu, 1999) and rinsed five times with sterile distilled water. The seeds were then pre-germinated in 15% water agar for 48 h at 26°C in an incubator. Germinated seeds were submerged in these suspensions, agitated on a shaker for 45 min. Sandy loam soil with pH 7.5 for each pot was disinfected in autoclave. Treated seeds were sown in soil moistened with tap water (3:1, vol/vol). Plastic boxes (11×17 cm) were filled with 200 grams of moist soil and 3 seeds were planted in 1-cm-deep holes.

One sclerot of *Sclertinia sclerotiorum,* approximately 0.7 to 1 cm was added near the sunflower seed and at last 2 cm of sterilized soil were added onto this collection. For the control treatment the seeds were dipped in 0.15% benomyl. The control (+) treatment included only the pathogen. Plants were grown in the greenhouse under day and night temperatures of 22±3°C with 16 h day light and 8 h night and 75% relative humidity. The pots were irrigated 3 times a week. For screening bioassays, the plants were removed after twenty days, the percentage of healthy plants was determined as described by Expert and Digat (1995). To compare the relative growth of the plants, plants were carefully desoiled through detailed rinsing in tap water, stems and roots cut apart and dried at 60°C for 48 h and weighed with accuracy. Each treatment was replicated three times and arranged in a completely randomized design.

Statistical analysis

Data were analyzed using analyzes of variance (ANOVA) with SAS software. Before the analyses were carried out, data on percentage of wilt severity were transformed using the log-transformations. Differences between treatments were determined by Duncan's Multiple Range Test (DMRT) at 5% significance level.

Acknowledgements

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