"Selection and utilization of Fluorescent Pseudomonads for enhancing production of sunflower crop in arid soil infested with Macrophomina phaseolina"

SUMMARY
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Sunflower is one of the fastest growing oilseed crop grown in India. Use of sunflower oil is in great demand as it trims down the incidence of cancer, hypertension, and the cholesterol in human beings. However, nowadays sunflower production is affected badly by several biotic and abiotic stresses. Amongst abiotic factors salinity is the main constraint that limits sunflower yield, whereas in biotic factors *M. phaseolina* is the phytopathogen which needs to be tackled in an ecofriendly and sustainable manner. *M. phaseolina* is fungal opportunist that likes to take advantage of stressed plant particularly in salinized regions and causes significant reduction in yields. Continuous use of chemicals in agriculture has resulted in degradation of soil fertility, formation of barren lands, disruption of soil ecology, development of resistant pathogens and ill effects on human health. Hence, there is an emergent need to develop biopreparations which can enhance plant growth, control phytopathogens, are effective even under stress conditions and above all are ecofriendly and hence lead to sustainability. Microbe-based formulations that can suppress the growth of phytopathogens and ameliorate the effect of abiotic stresses such as salinity are real alternatives to hazardous chemicals.

In the present study, 110 fluorescent Pseudomonads were isolated from Kanpur (Uttar Pradesh) and adjoining areas. Isolates were characterized on the basis of morphological, physiological, biochemical and molecular basis. The vernacular name 'fluorescent *Pseudomonas* group PF07-PF23 was coined for a group of 17 isolates from the rhizosphere of sunflower having characteristics that differentiate them from other members of fluorescent *Pseudomonas* spp. The isolates were monitored for stress tolerance capacity, plant growth promoting traits and biocontrol potential against *M. phaseolina*. On the basis of these attributes, two potent isolates, PF17 and PF23, were selected as they displayed maximum

salt, temperature and pH tolerance. In nature all the stress conditions occur simultaneously hence multi - stress tolerance assay was conducted to check the survivability of isolates. Isolates displayed survivability when multiple shocks (of salinity, temperature and pH) were given simultaneously.

Selected isolate PF23 was subjected to 16S rRNA gene sequence analysis, and was found to be *P. aeruginosa*. The 16S - 23S ITS region from PF17 gave a single amplicon of the size of 560 bp, which confirmed that the isolate was *P. fluorescence.* Protease, elastase and gelatinase activities of both the strains were checked so as to determine the pathogenicity. These initial level tests confirmed that both the strains were non-pathogenic, avirulent and hence safe to use. However, clinical trials will also be performed to completely ensure the safety before using these strains at large scale.

Strains were monitored for their plant growth promoting attributes and biocontrol potential against *M. phaseolina* under saline stress conditions. PF17 displayed production of diverse metabolites including P solubilization, Zn solubilzation, IAA production, siderophore, pyocyanin, HCN, chitinase and β-1,3 glucanase activity under saline conditions up to 600 mM NaCl. Which might have contributed in antagonizing *M. phaseolina* even, under saline conditions.

Results of plate assay clearly showed inhibition of *M. phaseolina* by PF17 up to 600 mM NaCl. *In planta* (tube) and *in vivo* (pot) studies were conducted with sunflower, under saline (125 mM NaCl) and non-saline (0 mM NaCl) conditions, both in presence and absence of phytopathogen *M. phaseolina*. Treatment of seeds with PF17 showed significant enhancement in plant growth parameters and suppression of charcoal rot disease incidence

even under saline conditions. PGP activities such as P solubilization, Zn solubilization, IAA and siderophore production displayed by PF17 even under high salinity might have participated in enhancing growth attributes of sunflower under saline conditions. Whereas, secretion of inhibitory metabolites such as HCN, pyocyanin, lytic enzymes might have helped in antagonizing phytopathogen in soil infested with it.

On the other hand *P. aeruginosa* PF23 showed production of EPS and SA up to very high salt concentrations. PF23 also displayed antagonism against *M. phaseolina* upto 500 mM NaCl in dual plate assay indicating the role of EPS and SA in controlling the pathogen. Purified EPS and SA from PF23 also showed inhibitory spectrum against *M. phaseolina* upto 500 mM NaCl. Phase contrast microscopy and SEM analysis from the zone of inhibition after treating with EPS and SA displayed perforation, curling, deformities and lysis of hyphae thereby confirming the role of EPS and SA in antagonism.

Analysis of EPS constituents by TLC revealed differences in the sugar components under varied salt concentrations. Under normal conditions (0 mM NaCl) glucose (Rf 0.42) was present as the major saccharide unit in the EPS hydrosylate, whereas EPS obtained under salt stress was composed of glucose (Rf 0.42), galactose (Rf 0.37), rhamnose (Rf 0.74), mannose (Rf 0.46), and trehalose (Rf 0.32). The FTIR spectrum of EPS produced by PF23 under different salt concentrations was analyzed and absorption bands gave typical polymeric structure of the carbohydrate containing hydroxyl group, methylene group, enol and amide group. The SEM of dried EPS obtained at 0 mM NaCl appeared to be smooth, glittering, spherical or ovoid exhibiting compact structure. However, photomicrographs of EPS obtained from 500 mM NaCl conditions appear polyhedral in shape with wrinkled surface. Multiple roles of EPS were illustrated by the study proved that at lower salt concentrations

EPS functioned as a biocontrol metabolite, but as the concentration of NaCl increased it started to behave more as an osmoprotant and when this EPS producing PF23 was pelleted on seeds and introduced in salinized soil it served as a biopriming agent.

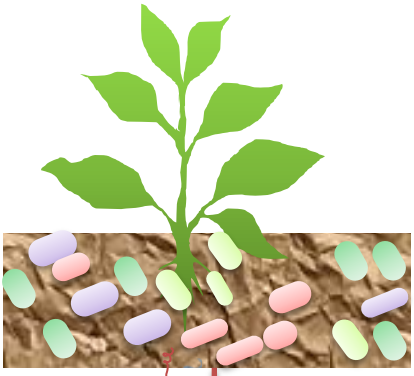
Mutational study was conducted to further confirm the role of EPS in disease suppression and stress amelioration. It was observed that EPS defective mutant $PF23^{EPS-}$ didn't display salt tolerance above 100 mM and showed significant reduction in EPS production in comparison to wild strain. Mutant was unable to maintain its growth under saline conditions and even it lost it antagonizing property above 100 mM. Thus a strong correlation could be observed between EPS production, salinity tolerance and biocontrol.

EPS producing strain $PF23^{EPS+}$ and its mutant $PF23^{EPS-}$ were checked for SA production ability. Strain $PF23^{EPS+}$ displayed SA production up to 500 mM NaCl whereas its defective mutant lost SA production above 100 mM salt concentration. HPLC analysis revealed single peak up to 500 mM NaCl concentration for $PF23^{EPS+}$ at different salinity levels that resembled to standard SA in respect to its retention time (4.502 min) however, there, was no peak recorded in case of mutant under saline conditions. It was found that the mutant strain was not only defective in EPS production but also displayed significant reduction in SA synthesis under non-saline (0 mM NaCl) conditions, followed by complete loss with increase in salinity, hence suggesting overlapping roles between these two metabolites (EPS and SA). Treatment of sunflower seeds with PF23 cells, purified SA and EPS (alone) or in combination with bacterial cells brought significant enhancement in growth attributes of under saline conditions in tube and pot assays.

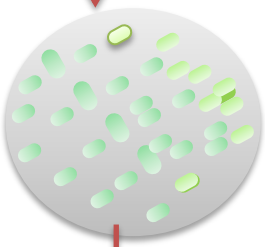
Both the strains were used to develop carrier based bioformulations. Amongst six carriers taken in the study (corn husk, coriander husk, coconut husk, charcoal, saw dust and talc), talc proved to be the best carrier in supporting the population of both the strains hence, talc based formulations were developed for field trials. Formulations were developed from normal cells (both PF17 and PF23), stressed cells (both PF17 and PF23), cell free culture supernatant (PF17 and PF23), metabolites (PF23), combination of metabolites (EPS + SA) and metabolites plus cells (PF23 + EPS or PF23 + SA).

It was observed that formulations developed from stressed cells and CFCS of PF17 and PF23 were effective in enhancing seed yield of sunflower under saline soil infested with *M. phaseolina*. However, formulation developed from metabolites (of PF23) or from cell plus metabolites proved to be best and were significantly similar in enhancing growth attributes of sunflower crop under saline conditions. Cell plus metabolites based formulation developed from EPS + PF23 and SA + PF23 enhanced seed yield by 195% and 180% respectively in comparison to untreated seeds. Combination of metabolites SA + EPS enhanced seed yield by 199.6%. The study, suggest that EPS and SA (as pure metabolites or in combination with bacterial cells) can thus be used to control the growth of phytopathogens and to enhance productivity of sunflower crop in salinized soils. The significant finding of the study encourages the use of bioformulations developed by blending PGP bacterial cells along with their metabolites.

Findings also suggest the use of such novel bioformulation (often optimization studies) for enhancing yield of such an important oilseed crop, sunflower, even under arid/semi-arid saline conditions. This ecofriendly biological product can also be used for reclamation of saline arid soils for enhanced productivity and food security.



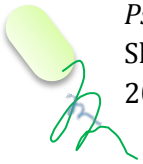
Rhizosphere of various plants containing diverse microflora



fluorescent bacteria isolated and monitored for stress tolerance (temperature, pH and salinity)

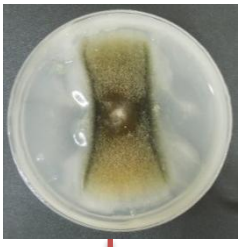
Two isolates PF17 and PF23 were selected on the basis of stress tolerance

Pseudomonas fluorescens PF17
Showed stress tolerance upto :
1400 mM NaCl , pH 3-13 & upto 50°C



Pseudomonas aeruginosa PF23
Showed stress tolerance upto:
2000 mM NaCl , pH 4-12 & upto 45°C

PF17 *inhibited M. phaseolina* upto 600 mM NaCl
Displayed PGP attributes like IAA, P solubilization, siderophore, pyocyanin, HCN, lipase, protease upto 600 mM NaCl



PF23 *inhibited M. phaseolina* upto 500 mM NaCl
Displayed PGP metabolites:
EPS and SA upto 2000 mM and 500 mM respectively

In planta tube and pot study conducted under non-saline (0 Mm NaCl) and saline (125 Mm NaCl) conditions taking sunflower as test crop)

cell based & metabolite based bioformulation developed fromPF17 and PF23

In vivo field trials conducted in semi-arid region, using diverse formulations

Metabolite based formulation brought maximum enhancement in plant growth attributes and disease suppressions

Salinized soil (control)

Untreated seeds

EPS+SA bioformualtion

EPS bioformulation

Figure 30: Summary of the work done