IDIOSYNCRASY OF LOCAL FUNGAL ISOLATE HYPOCREA RUFA STRAIN P2: PLANT GROWTH PROMOTION AND MYCOParasitism

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INTRODUCTION

Rhizospheric fungi have the ability to stimulate plant growth are designated as ‘Plant Growth Promoting Fungi’ (PGPF) (Hyakumachi, 1994). PGPF is non-pathogenic soil inhabiting saprophytes, which have been reported for growth promotion in several crop plants and providing protection against diseases (Shivanna et al., 1996). Such PGPF belongs to various genera, including Penicilium, Trichoderma, Fusarium, and Phoma. Few species of PGPF have been reported to trigger systemic resistance against numerous phytopathogens (Shoresh et al., 2005). Hypocrea rufa is a common inhabitant of the rhizosphere and decisively recognized as a bio-control agent of soil-borne plant pathogens (Harman et al., 2004). A praiseworthy amount of research has been focused on the mycoparasitic nature of H. rufa and its contribution in plant growth promotion. Antibiosis, competition, and mycoparasitism are the different mechanisms by which H. rufa controls plant-pathogenic fungi (John et al., 2010). The complex process of mycoparasitism requires the production of plenty of cell-wall-degrading enzymes, such as chitinases, cellulases, polysaccharidases, lyses, proteases, and lipases, which digest the fungal cell wall (Witkowska and Maj, 2002; Gruber and Seidl-Seiboth, 2012; P贵州省uan et al., 2013). The mechanism of PGF mediated plant growth promotion involves the production of various phytohormones like indole acetic acid (IAA) (Contreras-Cornejo et al., 2009), gibberellic acid and cytokinins (Salas-Marina et al., 2011). Moreover, plant growth promotion is also supported by the production of siderophore, mobilization of insoluble phosphate, induction of different plant pathogen defense-related enzymes (i.e. β-1, 3 glucanase, chitinases). Production of such enzymes contributed to their biocontrol characteristics (Dey et al., 2004; Chandler et al., 2008). Reduction in severity of plant diseases by application of H. rufa under field conditions was also reported by Hernosa et al. (2012). Field trials using talc-based bio-formulation of H. rufa was reported in India for the management of several soil-borne diseases applied through seed treatment and soil application (Jeyarajan, 2006; Mukherjee et al., 2012).

Groundnut (Arachis hypogaea L.) is an important oilseed crop in India. Gujarat is the principal producer state of groundnut in the country. The area and production of groundnut in the state found about 30.9 per cent and 37.1 per cent respectively in India (Swain 2013). The yield of groundnut is dropping by 25 per cent from 1,170 kg per hectare to 1,560 kg per hectare in Gujarat (SEA crop survey 2014). Various biotic and abiotic factors are responsible for this loss. There is 28 to 50% of mortality observed due to plant fungal pathogen (Ghewande et al., 2002). Various remedies like crop rotations, use of recommended chemical fungicide etc. are available to effectively control fungal disease. However, these types of strategies affect human health, environmental pollution, development of pathogen resistance to fungicide and the production cost (Patel et al., 2015b). Apart from all these strategies, an unconventional approach is by inoculating crop seeds and seedlings with plant growth promoting organisms (Patel et al., 2015a).

In spite of the well-documented history of H. rufa as a biofertilizer and biocontrol agent, very few researchers have aimed to test multiple traits from a single isolate simultaneously. Therefore, in the present study, multiple traits of PGPF were accessed in a local isolate H. rufa strain P2 along with its antagonistic activity against several fungal pathogens. In vivo plant growth promoting the activity of H. rufa strain P2 was determined using Arachis hypogaea L. as a test plant.

MATERIALS AND METHODS

Sample collection and Isolation

Farm soil sample was collected randomly at 15 cm soil depth using a cylindrical tube, from Anand, Gujarat (22°53’N, 72°96’E). One gram of soil was suspended in 9 ml of sterile distilled water. The serial aliquot of 0.1 ml was plated on selective medium, Rose Bengal agar with pentachloronitrobenzene (PCNB), and incubated at 28±2°C for 5-7 days. Isolate has shown green conidia was used further for experiments and identified using 18S rRNA gene sequencing. Pure culture was maintained and stored at 4°C on PDA.
Identification of isolate and morphological characterization

Isolate was grown for 5 days at 28±2°C in 100 ml of potato dextrose broth. Mycelia were collected by centrifugation. Fungal DNA extraction was done using GenElute™ Fungal Genomic DNA Extraction kit (Bangalore Genei, India). PCR amplification of 18S rRNA gene from the purified genomic DNA was carried out using the following primers (forward primer 5’-GGAAGTAAAAGTCGTAACAAGG-3’ and reverse primer 5’-TCCTCCGCGTTATGGATATGC-3’). Thermal cycler conditions involved an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 1 min, followed by holding at 4°C. Amplified gene product was sequenced at 1° BASE (Agile Life Science Technologies India Pvt. Ltd.). The BLASTn search program (http://www.ncbi.nlm.nih.gov) was used to look for nucleotide sequence homology. The gene sequence was submitted to GenBank under accession number KC351188. The similar sequences from European Molecular Database were aligned by ClustalW using MEGA 4.0 (Tamura et al., 2007) and a neighbor-joining (NJ) tree with bootstrap value 500 was generated. Morphological characteristics of the isolate were studied. Arrangements of conidia were visualized under a light compound microscope.

Selection of medium showing optimum growth of the fungal isolate

Sterile 100 ml Potato Dextrose Broth (PDB), Czapek Dox Broth (CDB) and Malt Extract Broth (MEB) were prepared in 250 ml flasks. All flasks were inoculated with the isolate. Plates were inoculated on the day of harvest. The plates were incubated at 28±2°C at 130 rpm. After completion of 3 and 6 days, the content of the flasks was filtered using pre-weighed Whatman filter paper No.1 and dried in hot air oven at 95°C until three constant weights of mycelia were obtained.

Assessment of plant growth promoting (PGP) traits

IAA production by isolate was determined using a method of Bano and Musarrat (2003). The quantitative estimation of a tri-calcium phosphate solubilisation by the isolate in the liquid Pikovskaya’s medium was estimated using a method described by Pikovskayas (1948). The pattern of change in the pH of the medium was recorded along with the tri-calcium phosphate solubilization using a pH meter. The concentration of the soluble phosphate was estimated from the supernatant using stannous chloride method after 13 days of incubation given by Usharani and Lakshmanaperumalsamy (2010). For ammonia production, the isolate was inoculated in peptone water and incubated for five days at 28±2°C. Biomass was separated through filtration and supernatant was used for estimation of ammonia production by the method given by Denmutskaya and Kalinichenko (2010). The concentration of ammonia was estimated against a standard curve of ammonium sulfate in the range of 0.1-1 µmol ml⁻¹. For the qualitative estimation of HCN production, Pictate assay was performed (Kang et al., 2010).

Biocontrol activity

The antagonistic capability of isolate P2 was assessed against prominent phytopathogens including Fusarium oxysporum (MTCC 3930), Alternaria alternata (MTCC 6572), Aspergillus niger (MTCC 2196), Sclerotium rolfsii (MTCC 6052) using dual culture technique (Patel et al., 2015b) with slight modification. Briefly, 10 mm diameter plug of the mycelial disc of a pathogenic fungus was taken from 6-8 day old culture plates and put it on PDA plates at one end in an inverted position and simultaneously, the other end was inoculated with the isolate. Plates were incubated at 28±2°C for five days. Inhibition percentage of a plant pathogen was calculated using formula % inhibition = [(I1-I2)/I1] x 100. Where I1 = radial growth of isolate, I2 = radial growth of a pathogen in dual culture experiments (Radial growth of the fungal strains were measured in mm).

Establishment of association between biomass and Chitinase enzyme

One agar plug of 10 mm size obtained from the four-day-old growth of H. rufa strain P2 was inoculated in 100 ml of sterile malt extract broth in 250 ml Erlenmeyer flasks and incubated at 28±2°C at 130 rpm. On each successive day, dry mycelial weight was determined as described earlier. The filtrate was stored at 4°C till assay was performed. Chitinase assay was performed with the slight modification of the method given by Lingappa and Lockwood (1962). Briefly, the assay mixture consists of 200 µl broth, 300 µl of 0.1 M sodium acetate buffer (pH 5.0) and 500 µl of 1.0% colloidal chitin and incubated at 37°C for 1 hour. Then a further 200 µl of 1 N NaOH were added to the each tube followed by centrifugation at 11000 rpm for 10 minutes at 4°C. 1 ml of Schales’ reagent (0.5 M Sodium Carbonate + 2.0 mM Potassium Ferricyanide) was added to the 500 µl supernatant was taken from each of tube followed by incubation in boiling water bath for 10-15 minutes. The absorbance was immediately measured at 420 nm using a spectrophotometer. The enzyme activity was calculated from a standard curve based on known concentrations of N-acetyl-glucosamine. One unit of chitinase activity was defined as the amount of enzyme that liberated 1µmol of N-acetyl glucose amine per hour.

In vivo pot study

The talc-based formulation of the isolated fungal strain was prepared (Soo and De Costa, 2012). Isolated strain was evaluated for its effects on the growth, yield and vegetative parameters of peanut (Arachis hypogaea L.). For ensuring complete sterilization, soil (medium black, pH 7.4) was sterilized at 121°C and 15 lbs for 1 h, thrice in autoclavable bags and 1 kg of soil was filled in a plastic pot of 16 cm diameter. Pots were watered at regular intervals. For bio-formulation application, the pot study was divided into two groups; (A) seed coating and (B) soil application. Different vegetative parameters like shoot length, numbers of leaves, numbers of branches, tap root length, lateral root length, fresh and dry root mass, fresh and dry shoot mass were measured after 15 days after sowing (DAS). Pot without application of talc-based bio-formulation of the isolate was used as a control.

Statistical analysis

Analysis of Variance (ANOVA) was carried out using triplicate value to identify a significant difference in each vegetative parameter between treated (seed coating, soil application) and non-treated seeds (control). Mean values of triplicates were compared at significance levels of 5%, 1%, and 0.1% LSD.

RESULTS

Morphological characteristics of the isolate

The isolate was grown on Rose Bengal agar amended with pentachloronitrobenzene (PCNB) as a green colony. One colony from the plate was transferred to PDA which was matured within 5 days at 28±2°C. Initially, isolate grew as a white color colony which turned into scattered blue-green or yellow-green patches after conidia formation. The colony was woolly and became compact; the patches were sometimes observed as concentric rings. The isolate was more readily visible on PDA and characterized rapidly by postulated branched conidiophores with lageniform phialides and green conidia born in slimy heads (Fig. 1) which were arranged repeatedly.

Identification of isolate

Amplified 18S rRNA gene product was sequenced at 1° BASE (Agile Life Science Technologies India Pvt. Ltd.). After performing BLASTn, 18S rRNA sequences of organisms showing maximum similarity were aligned by using ClustalW and an NJ tree was developed using software MEGA 4.0 with Bootstrap values based on 500 replications, which are listed as percentages at the branching points (Fig. 2). Gene sequence has been deposited in the GenBank nucleotide sequence database under the accession number KC351188. 18S rRNA sequence of H. rufa strain P2 showed maximum similarity with H. rufa strain W63 (JN935058).

Figure 1 Microscopic observation of mycelia and conidiophores under compound microscope

Figure 2 Phylogenetic analysis based on 18S rRNA gene sequences available from European Molecular Biology Laboratory (EMBL) library constructed after multiple alignments of data by ClustalW. Distances and clustering with the neighbor-joining method were performed using MEGA 4.0 software package.
Selection of the medium for the growth of organism

Liquid mediums viz., MEB, PDB, and CDB, were used to compare the growth of isolate which was measured as dry mycelia weight (DMW). Out of these three media, MEB best supported the growth of isolate. More than four-fold increase in DMW was obtained in MEB when compared to CDB and PDB on the third day. Even on the sixth day, DMW was more than two-fold higher in MEB (Fig. 3).

Plant growth promoting traits

The isolate was assessed for IAA production. *H. rufa* strain P2 in PDB medium showed the maximum of 72 µg ml⁻¹ production of IAA after 120 h (Fig. 4). To verify the relation between IAA production by the strain and the concentration of its precursor L-tryptophan, increasing the amount of this amino acid was added to the culture medium and production of IAA was estimated. The concurrent increase in IAA production was observed with increasing amount of L-Tryptophan supplementation (Fig. 4).

Isolate has shown the promising result for solubilisation of tri-calcium phosphate (Fig. 5). Isolate is capable of solubilizing inorganic phosphate by the production of organic acid. Isolate was capable of solubilizing maximum of 72 µg ml⁻¹ of tri-calcium phosphate after 11 days of incubation in the liquid Pikovskaya’s broth. Initial pH of the Pikovskaya’s medium prior to inoculation of the isolate was adjusted to 7.0 but the pH was decreased to 4.21 after 11 days of incubation indicating the production of organic acids. Isolate produced 2.35 mmol ml⁻¹ of ammonia in peptone water. However, HCN production was not detected.

Biocontrol activity

Isolate successfully inhibit potent plant pathogenic fungi viz., *F. oxysporum* (MTCC 3930), *A. alternata* (MTCC 6572), *A. niger* (MTCC 2196), *S. rolfsii* (MTCC 6052) under in-vitro conditions. The percentage inhibition varied with the pathogen. The percentage of inhibition was 57.6%, 48%, 34%, and 30% respectively for *F. oxysporum* (MTCC 3930), *A. alternata* (MTCC 6572), *A. niger* (MTCC 2196), *S. rolfsii* (MTCC 6052) (Fig. 6e).

Establishment of association between biomass and Chitinase enzyme activity

In this study, biomass increased up to the 6th day and later gradually depleted till the 13th day. Moreover, isolate showed characteristic antagonistic activity. Antagonistic activity is a key attribute for the bio-control agent. Chitinase is one of the important enzyme responsible for the inhibition of fungal pathogens. The cell wall of fungi made up of chitin. Therefore, corresponding changes in chitinase activity were estimated and related to the growth of the fungi to determine the association between a decrease in biomass and chitinase activity. In the present study, results showed a clear association between biomass and chitinase activity. There was an increase in biomass up to the 6th day when the chitinase activity was at the basal level, later on, chitinase activity rapidly increased with the consequent depletion of the biomass due to its own degradation by the enzyme. A similar trend was observed on day 11th (Fig. 7).
Several species of *Trichoderma* are widely known for their ability to be used as a bio-fertilizer and as a bio-control agent. Efforts are being made to commercialize isolates belonging to this genus. Therefore in the present study, the role of *H. rufa* strain P2 as biofertilizer and as an antagonist to several fungal phytopathogens was determined. Also, for commercialization, it is a very essential to know the optimum growth medium which can support maximum biomass production. Therefore, we also studied the biomass of the fungus produced in several commercially available growth media. MEB supported maximum growth of *H. rufa* strain P2. From the data obtained it is essential to understand the growth medium and the growth requirements of *H. rufa* strain P2.

Malt extract contains components like glucose, oligomers, inorganic salts, protein. Malt extract contains carbohydrates 91 g carbohydrates per 100 g (monosaccharides 10%, disaccharides 42-43%, oligosaccharides 38-39%), inorganic salts 1.8%, proteins 7.0%, and vitamins 0.2% of the total composition. This indicates that malt extract is not just providing carbon but also providing essential macro and micro nutrients. *H. rufa* strain P2 is saprophytic fungi that use a wide range of compounds as carbon and nitrogen sources. The carbohydrate components like glucose, oligomers, inorganic salts, protein. Malt extract contains carbohydrates 91 g carbohydrates per 100 g (monosaccharides 10%, disaccharides 42-43%, oligosaccharides 38-39%), inorganic salts 1.8%, proteins 7.0%, and vitamins 0.2% of the total composition. Therefore, malt extract supported the higher growth of *H. rufa* strain P2 by producing up to 10 g L\(^{-1}\) of biomass.

*H. rufa* is categorized as L-Tryptophan dependent IAA producer independent IAA producer (*Gupta et al., 1999*). So, here we proved this claim as we showed that *H. rufa* produces a higher concentration of IAA if the supplemented concentrations of L-tryptophan is also high. The isolate in this study showed several desirable features for PGPF and multiple action mechanisms, which...
suggest its potential for growth promotion in *Arachis hypogaea* L. One of these features can be observed as high levels of IAA produced after 12 h by fungus *H. rufa* strain P2, which are 12 fold high when compared to *T. atrovirete* (Gravel et al., 2007; Mukherjee et al., 2012) and about two-fold higher than *T. harzianum* in the range of 10 µg ml⁻¹ to 15 µg ml⁻¹. *H. rufa* strain P2 showed at least 2.5 fold higher production of IAA than these widely studied PGPR (*Bacillus* sp.). However, this may not be always in the plant rhizosphere, resulting in the production of biocontrol mechanism by PGPR. The deposition of IAA may vary according to the concentrations that are released in the root system and, depending on the plant variety.

The amount of P in the soil is 0.5% although only 0.1% is available to the plant. The solubilisation of Tri-calcium-phosphate observed in the present study is demonstrated to be related in decrease in P of the culture medium through the production of organic acids by *H. rufa* strain P2 by the utilization of glucose as a carbon source. Phosphate solubilising fungi like *Trichoderma* sp. and *Aspergillus* sp. produces different kinds of organic acids viz., lactic, maleic, malic, acetic, tartaric, citric, fumaric and gluconic acid (Akintokun et al., 2007). Deficiency of P in turn severely restricts plant growth and yield. *Trichoderma* isolates solubilizing inorganic phosphate (Pi) to P入户 (12.43 µg ml⁻¹), T. virnes PDDBC12 (9 µg ml⁻¹) and T. virnes PDDBC13 (8.83 µg ml⁻¹) was able to solubilize only 70% of the amount solubilized by *Bacillus megaterium* (12.43 µg ml⁻¹) (Rudresh et al., 2005). Superior ability to improve phosphate availability by *H. rufa* strain P2 used in the present study was evident through 1 to 6 fold more Pi solubilizing activity compared to previous reports. One of the important cause for the *H. rufa* strain P2 to be characterized as an antagonist to fungal phytopathogens is chitinase production which can easily degrade fungal cell wall. *Trichoderma* sp. is an antagonistic fungus, which prevents the crops from diseases viz. root rots, wilt, brown rot, damping off, charcoal rot and other soil borne diseases in crops. *Trichoderma* sp. suppresses more than 60 species of pathogens including *Pythium, Botrytis, Sclerotium, Fusarium, Ascochyta*, and *Alternaria* on different plants like cucumbers, tomatoes, cabbages, peppers, potato, coffee, sugarcane, apples, cauliflower, citrus, Chinese cabbage, sweet potatoes etc. Some of the fungal plant pathogens were inhibited by *H. rufa* strain P2 under present study is demonstrated (Fig. 7). *Trichoderma* sp. is an anamorph of *Trichoderma viride* through an Auxin mechanism (Mukherjee et al., 2012). The process of autolysis allows the remodeling of its growth by utilizing its own nutrients released into the medium by the action of chitinase on its own cell wall. During this process, cell wall polysaccharides are apparently exposed and consequently degraded. It is also possible that during other processes in fungal colony development, e.g. hyphal branching and fusion, the localized accessibility and de-protectition of chitin and another cell wall polysaccharides is a determining factor which plays important role in establishment of an association between chitinase, biomass production and shifting of biomass towards the production of chitinase (Gruber and Seidt-Seiboth 2012). In the present study, a similar trend was observed. Exo-chitinase can control the growth of *H. rufa* strain P2 in the degradation of cell wall made up of chitin (Solanki et al., 2011). *Trichoderma* sp. produces secondary metabolites including gliocton, glioerin and patabols with known antimicrobial activities that have been shown to act synergistically with lytic enzymes to enhance the destruction of host cell walls (Djovic et al., 2006; Mukherjee et al., 2012; Ruanpanun et al., 2012). Biofertilizers are useful in the formulations of agriculturally beneficial microorganisms. There are various ways of application i.e. seed, root or soil. Biofertilization improve nutrient status of the plant by various means including associative nitrogen fixation, phosphorus solubilization, siderophore production, altering the permeability and transforming nutrients in the rhizosphere resulting in the utilization by the plant. Biofertilizers can mobilize the nutrients available to improve the soil health by their biological activity. Biofertilizer colonizes the rhizosphere and promotes plant growth through increased supply of primary nutrients for the host plant. (Goswami et al., 2014). Some of the biofertilizers act as a phytostimulator which has capacity of the production of various phytohormones like IAA, GA, Cytokinins, and ethylene (Lugtenberg et al., 2002; Somers et al., 2004; Pindi et al., 2014). Biofertilizer plays a role like biopesticide by the production of antibiotics, siderophores, HCN (Vessey, 2003), while production of hydrolytic enzymes is also correlated with the mechanism of biofertilizer (Somers et al., 2004). Furthermore, the plant growth promotion by controlling phytopathogenic agents by means of acquired and induced systemic resistance (Chandler et al., 2008; In-vitro tests showed the presence of all these important traits which makes the local isolate P2 an efficient biocontrol as well as biofertilizer. Further, pot experiments confirmed the proposed scheme. *H. rufa* strain P2 used in the study showed 1.21-1.37 fold higher plant height after 14 DAS as compared to isolate studied by Nawangshih et al. (2012) for growth promotion and control the bacterial wilt disease in Peanut (Arachis hypogaea L.). Over the entire study, it can be confirmed that *H. rufa* strain P2 had several potentials which promote us use as a biofertilizer with the trait of mycoparasitism.

**CONCLUSION**

Isolate *H. rufa* strain P2 exhibited potential plant growth-promoting traits in vitro and could be a potential candidate for enhancing the growth of the plant and protect the plant from infection by pathogenic fungi. Seed application significantly improves vegetative parameters of *Arachis hypogaea* L. However, soil application showed clear edge with the more significant difference compared to other two treatments. Further, the salinity in the culture medium is a significant variable in the plant defense induction potential to evaluate candidature of isolate *H. rufa* strain P2 in field conditions.

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**REFERENCES**


http://dx.doi.org/10.1099/mic.0.052613-0

DOI: 10.1080/0322540999338319

http://dx.doi.org/10.1038/nrmicro797

http://dx.doi.org/10.1099/mic.0.05274-0

http://dx.doi.org/10.1094/PDIS.2003.87.1.4


http://dx.doi.org/10.1016/j.cropro.2010.08.004

http://dx.doi.org/10.1016/j.cropro.2010.06.004

DOI: 10.1099/mic.0.053629-0


http://dx.doi.org/10.1146/annurev.phyto.23.1.23

http://dx.doi.org/10.1016/j.jmbfs.2015.04.018

http://dx.doi.org/10.1016/j.micpro.2015.09.023


http://dx.doi.org/10.1007/s11274-010-0332-8

http://dx.doi.org/10.1139/w04-127

DOI: 10.4014/jmb.1101.01012


http://dx.doi.org/10.1016/0261-2194/96/00004-X

http://dx.doi.org/10.1094/PHYTO-95-0076

http://dx.doi.org/10.1080/09583157.2012.676025

http://dx.doi.org/10.1007/s13260-011-0188-x

http://dx.doi.org/10.1080/10408410490468786


http://dx.doi.org/10.1093/molbev/msm102

http://dx.doi.org/10.4314/jasem.v14i2.57887

http://dx.doi.org/10.1023/A:1026037216893

http://dx.doi.org/10.1023/A:1026037216893

http://dx.doi.org/10.1023/A:1026037216893

http://dx.doi.org/10.1023/A:1026037216893