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Isolation and Identification of Two Potassium Solubilizing Fungi from Arid Soil

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ABSTRACT

Keywords

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Five potassium solubilizing microorganisms were isolated from the arid soil of Jodhpur, India. Among them two fungal isolates RCKF7 followed by RCKF5 showed higher dissolution capacity towards feldspar, resulting in the release of more potassium compared to the others. Based on morphological characters and sequencing of ITS1–5.8S–ITS2 region the strains were identified as *Fomitopsis meliae* RCKF7 and *Aspergillus tubingensis* RCKF5. On further screening of the more potent strain RCKF7, it showed its ability to grow and solubilize potassium over a wide range of temperature (20 to 38 °C) and pH (5.0 to 10.0). The maximum potassium solubilization index was observed at 28 °C and pH 6.0. Capability of RCKF7 to solubilize phosphorus also rendered it an additional advantage. Furthermore the application of 500 ppm and 1000 ppm feldspar alone or in combination with RCKF7 resulted in more grain yield in wheat as compared to recommended dose of potassium fertilizer.

Introduction

After nitrogen (N) and phosphorus (P), potassium (K) is the third major essential macronutrients playing a key role in the growth and development of plants. In the world large areas of the agricultural land are deficient in potassium including three-fourth of the paddy soils of China and two-third of the wheat belt of Southern Australia (Meena *et al.*, 2014). The studies carried out on fertility status of Indian agricultural soils however, have shown that 21% of soils are low, 51% medium and 28% high in potassium. Hence about three-fourth agricultural soils require immediate attention (Hasan, 2002 and Meena *et al.*, 2016). As most of the potassium requirements in India is met through the imported fertilizer and with price varying between $460-625 per tonne this makes it a costly proposition. In the year 2009-10, India consumed 55.10 lakh tonnes of murate of potash) of which 42.38 lakh tonnes was used as fertilizer (Kinekar, 2011). It has been also projected that to meet the food demand of 1.3 billion Indians by 2020 the requirement of potassium by Indian agriculture would be 9.52 Mt (Pathak *et al.*, 2010). Also very low percentage of potassium present in the soil either naturally or when applied as synthetic fertilizers is available to plants as most of it is bound with other minerals, and is therefore unavailable to the plants (Goldstein, 1994 and Prajapati *et al.*, 2013). To increase the availability of potassium for plants, huge quantities of fertilizer are applied in the field on a regular basis. However, after application in the field a major proportion of these fertilizers is transformed to the insoluble forms necessitating continuous application (Kang *et al.*, 2002). Thus, the release of potassium from insoluble and fixed forms is an important aspect for increasing its availability in soil. The modification of various rock minerals in natural environments is mainly carried out by the action of water and organic acids produced by plant roots and microorganisms. Various potassium solubilizing microorganisms like *Bacillus*, *Enterobacter*, *Pseudomonas* and *Aspergillus* have been isolated and employed for their beneficial effect on plant growth promotion (Bagyalakshmi *et al.*, 2012; Prajapati *et al.*, 2013; Zhang and Kong, 2014 and Anjanadevi *et al.*, 2016). The different microorganisms and minerals from which they can release potassium have been reviewed recently (Sharma *et al.*, 2016). Large resources of good quality feldspar (8-10% potassium) in Rajasthan, India, could also be effectively utilized by native potassium solubilizing microorganisms. Therefore the present work was conducted to isolate and test feldspar solubilization by strains from arid soil and to assess its response on growth of wheat.

Materials and Methods

Isolation and purification of microorganisms from arid soil

Soil samples were collected from different locations in the arid environment of ICAR-Central Arid Zone Research Institute, Jodhpur in 2014. Ten-fold serial dilutions of soil samples were prepared in sterilized distilled water, and 0.1 mL of diluted samples was spreaded on the surface of nutrient agar (0.3% beef extract, 0.5% peptone, 0.5 % NaCl, and 1.7% agar) and potato dextrose agar (20% potatoes, 2% dextrose,1.7% agar) (HiMedia, India) for isolation for bacteria and fungi (Kasana *et al.*, 2008). Plates were incubated at 28^{o}C for 48 hours for bacteria and 96-120 hours for fungi. Morphologically different colonies appearing on the plates were purified on the respective medium for bacteria and fungi.

Screening of microorganisms for potassium solubilization

The microorganisms were screened for potassium solubilizing activity on Aleksandrov agar medium (Hu *et al.*, 2006) having: 0.5% glucose, 0.05% magnesium sulfate heptahydrate, 0.0005% iron chloride, 0.01% calcium carbonate, 0.2% calcium phosphate, 0.2% feldspar (potassium aluminum silicate) and 1.8% agar; by spot plate method.

Identification of fungal isolates

Morphological studies were conducted by growing the strains on potato dextrose agar plates and incubating at 28 ^{o}C. The visual and microscopic examination of the fungal growth was conducted. For molecular identification the fungal strains RCKF5 and RCKF7 were cultivated in 100 mL of liquid medium at 28 ^{o}C under continuous agitation at 150 rpm. Mycelia from 96 hour grown cultures harvested and DNA was extracted. The DNA was amplified by using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. The PCR was initiated by incubating the reaction mixture at 94^{o}C for 3 min, followed by 35 cycles of 1 min at 94 ^{o}C. The reaction was annealed at 55 ^{o}C for 1 min and terminated with extension and final steps consisting of 1 min at 72 ^{o}C and 10 min at 72 ^{o}C. The amplified gene products were electrophoresed on 1.2% agarose gel and desired band of about 600 bp was excised and purified using Qiaquick Gel Extraction Kit

(Qiagen, Germany). Nucleotide sequencing of the genes was done by using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and 3130xl Genetic Analyzer (Applied Biosystems). The BLASTN program http://www.ncbi.nlm.nih.gov/BLAST/; National Center for Biotechnology Information, Bethesda, MD) was used for homology searches with the standard program default.

Assessment of potassium solubilization potential of isolates

Effect of temperature and pH on potassium solubilization

To check the effect of temperature on potassium solubilization the fungal culture was inoculated on Aleksandrov agar medium and plates were incubated at three different temperatures 20, 28 and 38 °C in BOD incubator. To study the effect of pH on potassium solubilization Aleksandrov agar medium plates of varying pH ranging from 5.0 to 10.0 were prepared and inoculated with culture. Plates were incubated at 28 °C and 38 °C in BOD incubators. Solubilization index was calculated using the formula given below

$$\text{Solublizing index} = \frac{\text{Colony diameter + clearing zone}}{\text{Colony diameter}}$$

Quantitative estimation of potassium solubilization

Feldspar was added to the liquid Aleksandrov medium as the sole potassium source to test the ability of the isolate to solubilize it. Quantitative estimation of potassium solubilization was carried out in Erlenmeyer flasks containing 100 mL of Aleksandrov medium, and inoculated in triplicate with RCKF7. Autoclaved, uninoculated medium served as controls. The flasks were incubated at 38 °C in a BOD incubator. The supernatant obtained by filtering the culture using Whatman filter number 42 was used to assay the solubilized potassium using atomic absorption spectrometry (Manib *et al.*, 1986).

Pot experiment

The fungus RCKF7 was grown in potato dextrose broth for 7 days. Four gram of wet mycelium was crushed in 100 mL of water. The plastic pots (22 cm top diameter, 14 cm bottom and 22 cm height) were filled @5 kg pot^{-1} sandy soil (pH 8.4, electrical conductivity 0.29 dS m^{-1}, organic carbon 2.1 g kg^{-1}, available N 94.5 kg ha^{-1}, available P 10.60 kg ha^{-1}, available K 236 kg ha^{-1} and available sulfur 8.66 ppm.

Feldspar was added to the soil in pots as per the treatments and thoroughly mixed. Seeds of wheat were soaked in the water containing the crushed mycelium for 30 minutes and seven seeds were sown 2 cm deep in each pot. Performance of wheat was assessed under following nine treatments with three replications. Soil alone, soil + recommended dose of NP, soil + recommended dose of NPK, soil + recommended dose of NP + 250 ppm Feldspar, soil + recommended dose of NP + 500 ppm Feldspar, soil + recommended dose of NP + 1000 ppm Feldspar, soil + recommended dose of NP + 250 ppm Feldspar + RCKF7, soil + recommended dose of NP + 500 ppm Feldspar + RCKF7 and soil + recommended dose of NP + 1000 ppm Feldspar + RCKF7.

The pots were irrigated at regular interval and after 15 days of germination plants were thinned to five plants per pot. The rate of net photosynthesis was measured in two uppermost fully expanded leaves of intact plants using CIRAS-2 portable photosynthesis system. The measurements were made on three plants in each treatment between 10-12 hour at 67 days of sowing. In vivo estimation of nitrate reductase activity using leaf disc from same leaves was done following the method of Jaworski, 1971. The crop was harvested after 93 days of sowing and data of dry shoot weight, weight of spikes and grain yield per pot were recorded.

Screening of potassium solubilizing microoganisms for phosphate solubilization

Potassium solubilizing isolate RCKF7 was also screened for phosphate solubilizing activity on Pikovskaya's medium: 1.0% glucose, 0.5% tricalcium phosphate, 0.05 ammonium sulphate, 0.02% potassium chloride, 0.01% magnesium sulfate heptahydrate, traces of manganese sulphate and iron sulphate, 0.05% yeast extract and 1.8% Agar (Himedia) by spot inoculation. A clear zone around the colony indicated the potassium and phosphate solubilization.

Results and Discussion

Isolation and purification of microorganisms from arid soil

Twenty five bacterial and fifteen fungal isolates were selected and purified based on morphological characteristics from arid environment soil samples collected from Jodhpur. Among these isolates three fungal and two bacterial isolates showed potassium solubilization.

Results suggest the existence of potassium solubilizing fungi in desert soil environments. Potassium solubilization on mica powder containing medium plates have been reported earlier (Parmar and Sindhu, 2013).

Potassium solubilizing isolates from various rhizospheric soils samples have also been reported from Inceptisol and Alfisol (Maurya *et al.*, 2014).

Screening of microorganisms for potassium solubilizations

Strains RCKF7 followed by RCKF5 which formed larger zone of clearance on Aleksandrov agar medium were selected as potent potassium solubilizer and used for further studies (Fig. 1).

Identification of fungal isolates

The culture on potato dextrose agar showed white mycelium for two-three days which became black as conidia develop in case of RCKF5. The guttulate hyphae and uniserate conidiophores indicated its affiliation to genus *Aspergillus*. Based on morphological characters the strain RCKF7 was identified as belonging to genus *Fomitopsis* (Kang *et al.*, 2002). The upper surface of colonies appeared white on potato dextrose agar which was also white on the reverse side, pigment absent. The texture of the colonies on potato dextrose agar was powdery with velvety appearance. Spore formation did not occur on potato dextrose agar after prolonged incubation (Fig. 1). Further identification and phylogenetic relationship studies of RCKF7 and RCKF5 were conducted based on the sequenced ITS1–5.8S–ITS2 region. The sequences were also submitted to NCBI with accession numbers KT718002 and KT718003 respectively. In case of RCKF5 a homology of 99.83% was found with *Aspergillus tubingensis* strain CBS 134.48 with a published species in literature (Accensi *et al.*, 1999). In case of RCKF7 a homology of 97.7% was found with *Fomitopsis meliae* voucher SRM-209 with a published species in the literature (Ortiz-Santana *et al.*, 2013). Though, RCKF7 showed homology of only 97.7% but was identified as *Fomitopsis meliae* RCKF7, it may represent new species of genus Fomotopsis. The sequences of the isolate were then aligned and compared with previously published sequences of the species from genus *Fomitopsis* and *Aspergillus* with published names, and neighbor-joining phylogenetic trees were constructed using MEGA6 (Tamura *et al.*, 2013). In phylogenetic analysis the strain RCKF7 clustered with *Fomitopsis meliae* SRM-209-KC585351 (Fig. 2a) whereas, in case of the strain RCKF5, it clustered with *Aspergillus tubingensis* CBS 134.48 AJ223853 (Fig. 2b). Though bacteria belonging to various genera have been reported for solubilization of potassium, however in case of fungi the strains belonging only to the genus *Aspergillus* (Lopes-Assad *et al.*, 2010), have been reported for solubilization of potassium. Mostly, *Fomitopsis* species have been isolated as endophytes from various sources like *Fomitopsis cf. meliae*, *F. cf. ostreiformis*, *F. cf. pinicola* were isolated from the oil palm, *Elaeis guineensis*, at an oil palm plantation in Trang Province, Thailand (Rungjindamai *et al.*, 2008 and Pinruan *et al.*, 2010), *Fomitopsis cf. meliae* isolated from the *Bacopa monnieri* (Katoch *et al.*, 2014), but *Fomitopsis* sp. PS 102 was isolated from the ground soil at Taegu, South Korea (Kang *et al.*, 2002). The strains belonging to genus *Fomitopsis* have been reported for phosphate solubilizing ability and potent cytotoxic and antimicrobial properties (Kang *et al.*, 2002 and Katoch *et al.*, 2014), but this is first report on potassium solubilization by fungi belonging to genus *Fomitopsis*.

Potassium solubilization at different temperatures and pHs

Fomitopsis meliae RCKF7 was isolated from desert soil and has the ability to grow and solubilize potassium over wide range of temperature and pH. *Fomitopsis meliae* RCKF7 was grown at different temperatures on Aleksandrov agar medium for solubilization of potassium. It showed highest growth and larger clearing zone at 38 ºC followed by 28 ºC and 20 ºC (Fig. 3). This reflects the adaptability of the isolate to wide range of temperature that also naturally occurs in arid soils. The maximum solubilization index was observed at 28 ºC followed by 38 ºC and 20 ºC and (Fig. 4). Among various bacterial strains isolated from rhizosphere soil of wheat two strains WPS73 and NNY43 showed maximum potassium solubilization at 25 and 30 °C respectively, whereas other bacterial strains showed significant solubilization in the temperature range of 25 °C to 35 °C (Parmar and Sindhu, 2013).

The quantative estimation of potassium release from feldspar source at 38 ºC using atomic absorption spectrometry was estimated to be 7.1 mg L^{-1}. Studies conducted recently on potassium solubilization have shown that potassium release from feldspar ranged from 0.13 to 12.25 mg L^{-1} by different microbial isolates (Setiawati and Mutmainnah, 2016).

As most of the fungal isolates generally prefer acidic pH for their growth hence to observe the effect of pH on K solubilization strain RCKF7 was inoculated on Aleksandrov agar plates of different pH and incubated at two temperatures of 28 and 38 ºC. The fungus showed better growth at acidic pH 5 which declined with increase in the pH, with minimum growth at pH 10 as evident from the colony size (Fig. 4). The solubilization index was observed in the range of 2.06 to 2.15 and at both the temperatures the maximum solubilization index was observed at pH 6 (Fig. 4).

As agriculture is practiced under various agro climatic conditions with lot of variation in temperature and soil parameters, hence strains capable of potassium solubilization under wide range of temperature and pH have wider applicability. Furthermore, along with potassium solubilization strain RCKF7 also showed the solubilization of phosphorus rendering it an additional advantage.

Effect of application of feldspar and potassium solubilizing fungus on wheat

Application of feldspar and potassium solubilizing fungi resulted in an increase in shoot dry weight, weight of spikes and grain yield (Table 1). Maximum shoot dry weight of 48.7 g was obtained with application of 1000 ppm of feldspar, while maximum weight of spikes and grain yield of 22.43 and 15.77 respectively were obtained with application of 500 ppm of feldspar and RCKF7. Application of 500 ppm and 1000 ppm feldspar alone or in combination with RCKF7 resulted in more grain yield as compared to recommended dose of potassium fertilizer (Table 1). This is in line with the earlier report wherein authors reported that use of imbalanced fertilizers, including potash leads to a reduction of soil potash reserves and results in yield losses (Tan *et al.*, 2005). Microorganisms capable of dissolving potassium from mineral and rocks influence the plant growth and have both economic and environmental advantage. Significant increases in yield due to application of potassium solubilizing microorganisms have been also reported in *Capsicum annuum* (Supanjani *et al.*, 2006) and sudan grass (Basak and Biswas, 2009). This could be either due to more uptake of potassium as in tobacco (Zhang and Kong, 2014) and okra (Prajapati *et al.*, 2013).

Table.1 Effect of feldspar solubilizing fungi RCKF 7on wheat under pot condtions

Treatments	Shoot dry weight (g) /pot	Weight of spikes (g) /pot	Grain yield (g) /pot
Soil (Control)	28.53	14.00	9.50
Soil +NP	39.90	18.50	11.97
Soil +NP+K	44.17	20.00	13.43
Soil+Felds (250ppm) +NP	30.93	16.93	11.73
Soil+Felds (500ppm) +NP	46.67	22.00	15.57
Soil+Felds (1000ppm) +NP	48.70	21.13	14.17
Soil+Felds (250ppm)+RCKF7+NP	31.83	18.60	12.40
Soil+Felds (500ppm)+RCKF7+NP	41.90	22.43	15.77
Soil+Felds (1000ppm)+RCKF7+NP	38.63	21.40	14.90

Fig.1 Pure culture of RCKF7 on a) potato dextrose agar and b and c) potassium solubilization on Aleksandrov agar by RCKF7 and RCKF5 as indicated by hallow formation around the growth

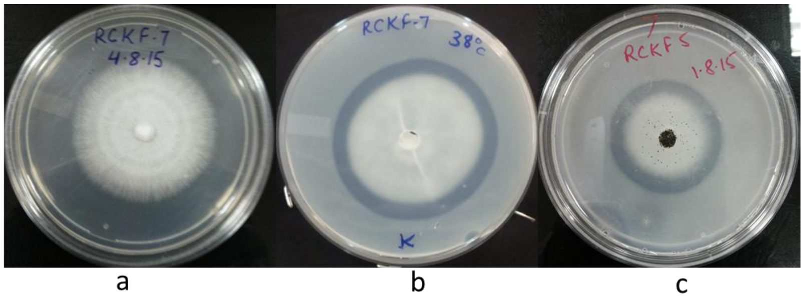


Fig.2a Phylogenetic tree based on ITS1–5.8S–ITS2 region sequences, drawn using the neighbor joining method and showing the relationship between *Fomitopsis meliae* RCKF7 and species from genus *Fomitopsis*. The sequences were downloaded from NCBI database. *Aspergillus tubingensis* was used to root the tree. Bar, 0.1 substitutions per site. Evolutionary analyses were conducted in MEGA6

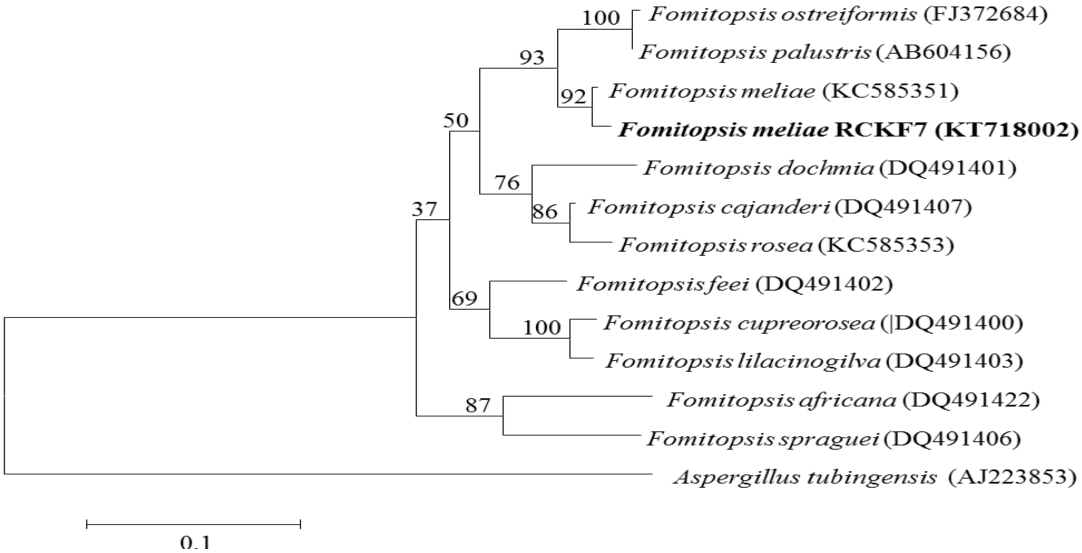


Fig.2b Phylogenetic tree based on ITS1–5.8S–ITS2 region sequences, drawn using the neighbor joining method and showing the relationship between *Aspergillus tubingensis* RCKF5 and species from genus *Aspergillus*. The sequences were downloaded from NCBI database. *Fomitopsis* africana was used to root the tree. Bar, 0.05 substitutions per site. Evolutionary analyses were conducted in MEGA6

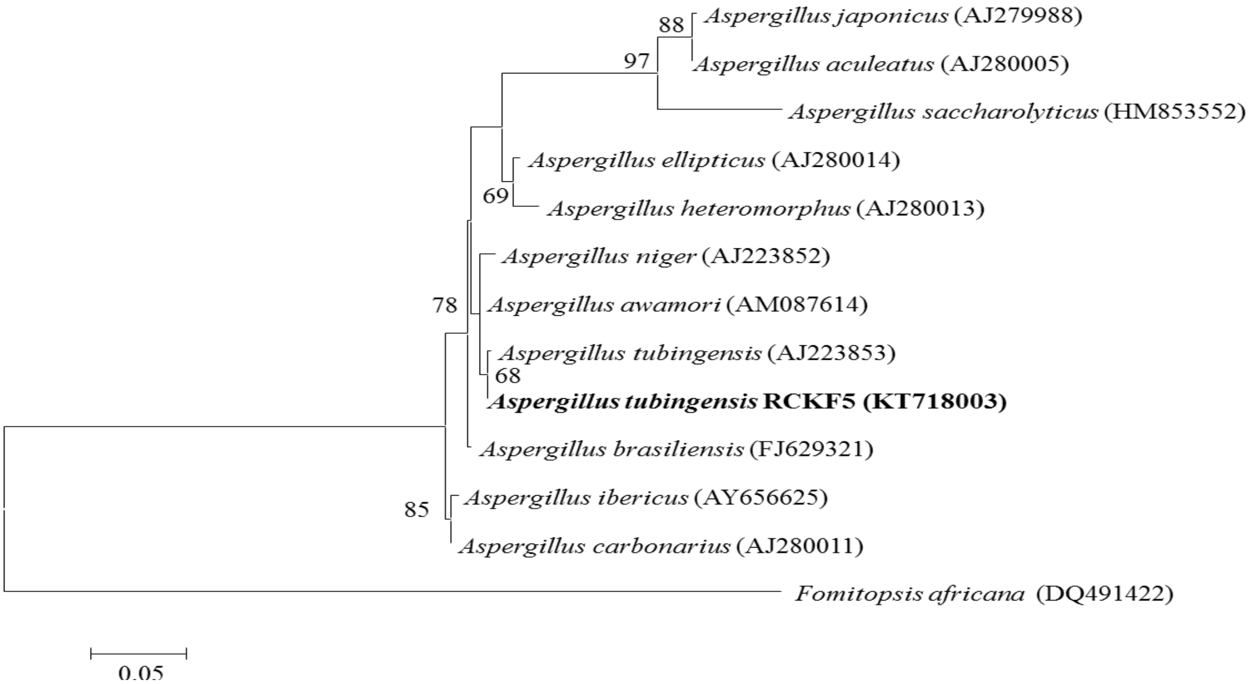


Fig.3 Potassium solubilization by RCKF7 at different temperature

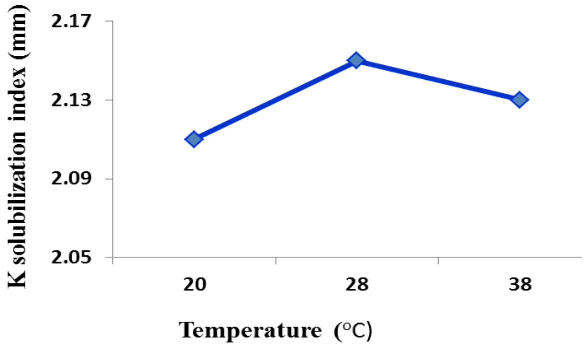


Fig.4 Potassium solubilization by RCKF7 at different pH

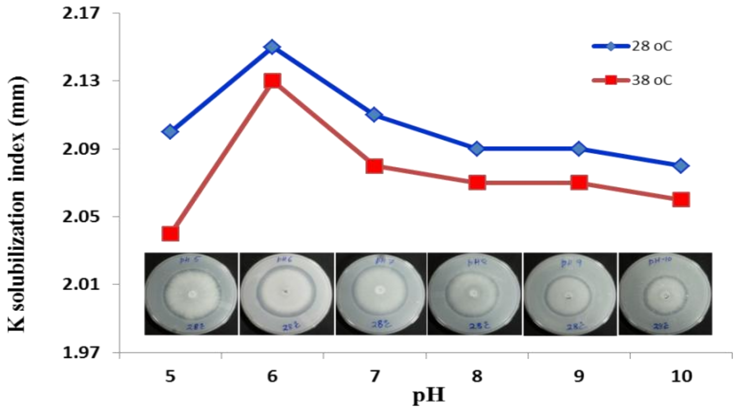
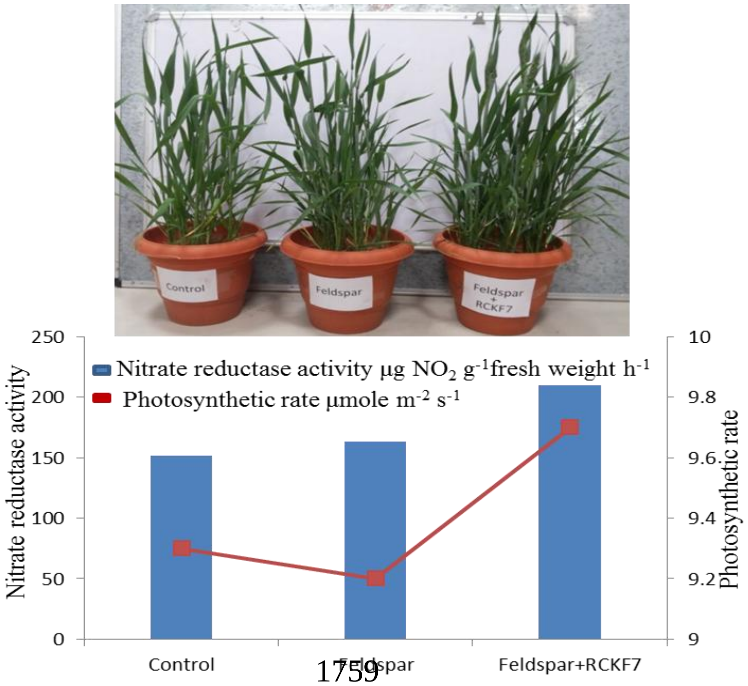


Fig.5 Effect of inoculation with potassium solubilizing fungi RCKF7 on nitrate reductase activity & photosynthetic rate of wheat under pot conditions



Release of potassium through use of potassium solubilizing microorganisms from feldspar and other minerals rocks provide possibility of potassium solubilization (Hassan, *et al.*, 2010 and Anjanadevi *et al.*, 2016.) In our work better growth under feldspar alone or feldspar along with RCKF7 (Fig. 5) compared to control corroborated with higher nitrate reductase activity and photosynthetic rate (Fig. 5). This could be possibly through regulation of more carbon reduction cycle enzymes besides photorespiration through stomatal activity by potassium (Page *et al.*, 2006). In control inadequate potassium may also adversely affect phloem transport (Cakmak *et al.*, 1994a and 1994b) which in turn limits the carbon supply required for continuous nitrogen assimilation and growth. Nitrate reductase has also been reported to significantly decrease under potassium deficient conditions (Armengaud *et al.*, 2009). Suboptimal level of potassium also results in buildup of photosynthate in leaves due to adverse effect on transport and subsequent reduction in rate of photosynthesis.

In conclusion, this is the first report of solubilization of feldspar by a fungus (RCKF7) belonging to the genus *Fomitopsis*. Its ability to grow and solubilize feldspar at wide range of temperature and pH shows that this fungus may serve as good feldspar solubilizer when inoculated into the arid soils where potassium availability is otherwise low due to poor soil structure. In arid zone where resource limited farmers's often overlook importance of potassium and prioritize more in favour of nitrogen and phosphorus, application of feldspar along with potassium solubilizing microorganism is likely to give better yield over a long period.

Further studies needs to be conducted in order to find out the actual level of solubilization while considering the potassium consumed by the fungus and its efficiency in solubilizing the feldspar under field conditions.

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