A preliminary study on L-asparaginase from mangrove detritus-derived fungi and its application in plant growth promotion

Varada S. Damare*, Kiran G. Kajawadekar

Department of Microbiology, Goa University. Taleigao Plateau, Goa 403206, India
*Corresponding author, email: chimulkavarada@gmail.com

Abstract

L-asparaginase enzyme has wide applications in medicine, food industry and developing biosensors. The present study was carried out to investigate the plant growth-promoting characteristics of L-asparaginase, an aspect that is poorly studied. Two filamentous fungi, *Peroneutypa* sp. GU-S and *Lecanicillium* sp. GU-G, derived from mangrove detritus in Goa, India, produced L-asparaginase enzyme in laboratory conditions which was active at room temperature, and 37 °C. A higher amount of enzyme was produced under aeration than without aeration. The optimum pH for asparaginase activity was 8. The enzyme isolated from *Peroneutypa* sp. GU-S was more stable at lower pH 4, and thus further processed. The enzyme was produced using economical sources, i.e., powdered soya and grass by solid-state fermentation. Germinating seeds of pea, *Pisum sativum* inoculated with crude enzyme extract produced a better quality of seedlings than the seedlings from un-inoculated seeds. The shoot and the root length were 1.2 and 2.2 times greater in the test than in control plants, within twenty days of incubation. This study demonstrates plant growth-promoting ability of the L-asparaginase, which can be further studied on other plants.

Keywords: *Lecanicillium*, *Peroneutypa*, *Pisum sativum*, plant growth-promoter, solid-state fermentation

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Introduction

Mangrove environment supports a wide variety of fauna and flora (Kathiresan and Bingham 2001). The microbial community composition in mangroves is heterogeneous and a good source of bioactive compounds (Bandaranayake 2002, Castro et al. 2014). Mangrove-derived fungi are known to produce enzymes which degrade a wide range of substrates, thus playing a vital role in nutrient cycling in marine environment (Raghukumar et al. 2004, D'Souza-Ticlo et al. 2006, Thatoi et al. 2012). Thatoi et al. (2012) discussed the possibility of using mangrove-derived fungi and their enzymes in industries such as pulp and paper, detergent, sewage treatment. L-asparaginase enzyme produced by fungi is used in biomedical and food industry (Cachumba et al. 2016). However, there are limited reports on L-asparaginase from mangrove ecosystem (Shome and Shome 2001, Gupta et al. 2009, Rajamanickam et al. 2011).

L-asparaginase enzyme is well known for its antineoplastic properties and has been successfully used in chemotherapy for the treatment of acute lymphoblastic leukaemia (Sanaa et al. 2012, El-Naggar et al. 2014). L-asparaginase inhibits the growth of actively growing tumours and shrinks them by depriving their cells of L-asparagine, which is an essential amino acid (Narta et al. 2007). Besides its use in chemotherapy, L-asparaginase finds application in

the treatment of autoimmune diseases as well as infectious diseases due to its immunosuppressive and anti-inflammatory properties (Vimal and Kumar 2018). In recent years, this enzyme has found application in food industries to breakdown L-asparagine, which would otherwise get converted to toxic acrylamide during baking or frying of food (Morales et al. 2008). Novozymes and DSM have commercialized L-asparaginase from *Aspergillus niger* and *A. oryzae*, respectively, for their application in dough-based food industries (Morales et al. 2008).

L-asparaginase is poorly studied for its application in plant growth promotion. It is essential for the development of seeds/ grains in plants. It breaks down asparagine into aspartic acid and ammonia, and these two byproducts provide nutrition for the plant and also serve as a precursor of other amino acids that stimulate plant growth. The amount of asparagine and glutamine determines the food production in plants as these are the two amino acids that are translocated through the phloem (Credali et al. 2013, Yabuki et al. 2017). Hence, the supply of L-asparaginase is crucial for plants. Higher plants have genes for the production of this enzyme (Credali et al. 2013). Nevertheless, the enzyme could also be obtained from external sources. Microorganisms that inhabit as endophytes produce several growth-promoting factors such as gibberellins, indole acetic acid; that promote the growth of their host plant (Bilal et al. 2018, Shah et al. 2019). Endophytic fungi from seaweeds reportedly produce L-asparaginase enzyme (Thangavel et al. 2013). However, there are no studies on the plant growth-promoting ability of L-asparaginase from a fungal source.

The present study aimed to investigate whether L-asparaginase provided from an external source plays any role in enhancing the growth of seedlings. The source here was fungi isolated from mangrove detritus. Since mangrove environment and microorganisms living therein are subjected to environmental fluctuations due to tidal influence, these microorganisms produce enzymes that are stable under fluctuating environmental conditions (Kathiresan and Bingham 2001, Thatoi et al. 2012). The focus of the present study was therefore to isolate a fungus from mangrove environment that can produce L-asparaginase enzyme, having a potential application in plant growth promotion. Further, it was important to determine whether the enzyme produced by the fungus is stable at wide environmental conditions and could be used for commercial production. The latter is possible if the enzyme is produced under economical conditions such as growing the fungus in presence of easily and abundantly available substrates such as various agro-waste products or waste products of different industries (Dias et al. 2015).

Materials and methods

Isolation of fungi

Sediment sample laden with leaf detritus, as evident by its black colour, was collected from Ribandar mangroves along the Mandovi river, Goa, India. One gram of the sample was aseptically inoculated in 100 ml of Minimal Salt Medium (MSM) amended with 1 % L-Asparagine in flasks and was enriched for five days. A few drops of phenol red indicator were added to the medium which turned the medium colour from orange-yellow to pink after the incubation period. One ml of this enriched broth was serially diluted up to 10^{-3} using sterile saline, of which 0.1 ml was spread plated on MSM and incubated for five days.

Growth, maintenance and qualitative examination of fungal isolates for L-asparaginase enzyme production

MSM containing 6 gL^{-1} $Na_2HPO_4.2H_2O$, 3 gL^{-1} KH_2PO_4, 5 gL^{-1} NaCl, 2 mL of 1M $MgSO_4.7H_2O$, 1 mL of 0.1M $CaCl_2.2H_2O$ and supplemented with 1% Glucose, 1% L-

asparagine and 2.5% phenol red (as a pH indicator) was used to maintain the fungal isolates and check their ability to produce L-asparaginase enzyme.

Taxonomic identification of fungal isolates

The fungal ITS region was amplified from the genomic DNA using ITS1 and ITS4 primers (White et al. 1990). For this, the biomass of the isolates was extracted after seven days of growth in MSM broth and DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep kit (Cat. No. D6005, Zymo Research). The PCR conditions were as follows; 94 °C for 4 min, followed by 30 cycles of 94 °C for 50 s, 52 °C for 45 s and 72 °C for 1 min, with a final elongation step of 72 °C for 7 min. This was followed by purification of the PCR products using Wizard SV Gel and PCR Clean Up kit (Promega, Cat. No. A9282) and DNA sequencing using a 3130xl Genetic Analyzer (ABI Sequencer 2200), Applied Biosystems by capillary electrophoresis at CSIR-National Institute of Oceanography, Goa, India.

Quantitative estimation of enzymatic activity

L-asparaginase activity was determined by measuring the amount of ammonia released by using Nessler's method described by Wriston and Yellin (1973). Fungal isolates were grown in M9 medium broth with 1 % L-asparagine. The assay was performed by following the method described by El-Naggar et al. (2016). The reaction mixture containing 1.5 ml of 40 mM L-asparagine was prepared in 50 mM Tris-HCl buffer, pH 8.6 and mixed with 0.5 ml of the enzyme to make up the volume to 2 ml. The tubes were incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 1.5 M Trichloroacetic acid (TCA). Precipitated protein was removed by centrifugation at 11,000 ×g for 5 min, and the liberated ammonia in the supernatant was determined by Nessler's method. Briefly, 1 ml of Nessler's reagent was added into tubes containing 0.5 ml clear supernatant and 7 ml distilled water followed by incubation at room temperature for 20 min. After incubation, the formation of yellow colour indicated the presence of ammonia and the appearance of brown colour indicated the release of the high amount of ammonia. The UV-spectrophotometric analysis was performed at 480 nm. The liberated ammonia was calculated according to the standard curve of ammonia (Shifrin et al. 1974). This method of enzyme assay was followed throughout the present study.

Activity of enzyme= [(µmol of ammonia liberated)(initial volume of mixture in ml)]/[(volume of enzyme mixture used in final reaction in ml)(incubation time)(volume of enzyme ued)]

Effect of aeration on enzyme production

The culture flasks were incubated at RT for 72 h on a shaker at 150 rpm and also under static conditions as a control (no aeration) to check the effect of aeration. An aliquot of 1 ml was taken every 24 h till day 7 to determine the enzyme activity.

Effects of different conditions on enzymatic activity

Effect of temperature: Enzymatic activity was examined at RT and 37 °C, to check if the enzyme produced at RT can show activity at two different temperatures. For this, the fungal isolates were grown under two conditions, with and without aeration, as mentioned above.

Effect of pH: Optimum pH for activity was examined for the fungal isolates by first growing them in MSM broth with 1 % of L-asparagine for three days. The culture broth was filtered through Whatman filter paper. The filtrate was used for determination of enzyme activity at different pH viz. 4, 5, 6, 7, 8, 9, and 10 at RT.

Effect of pH on enzyme stability: Enzyme stability of the fungal isolates was determined at pH 4, 8 and 8.6. The crude enzyme was incubated in the respective pH at RT and under refrigerated conditions (4 °C) for 24 h, and the activity was determined at 7, 12 and 24 h.

Determination of inexpensive sources for growth of the fungi

Different substrates such as soya chunks ground to a coarse powder, wheat bran, sugar cane bagasse and dried grass were used to grow the fungal isolate GU-S for production of L-asparaginase through solid-state fermentation (SSF). Shatavari powder (obtained from the local market) to a concentration of 0.05 % was used as an inducer in place of L-asparagine. Shatavari is an extract of dried roots of *Asparagus racemosus* herb and is widely used as Ayurvedic medicine in India. Inoculum preparation was carried out by growing the isolate in MSM broth at RT for three days under static condition, followed by sub-culturing in freshly prepared MSM broth. After three days of incubation, the fungal mycelia were removed with the help of sterile forceps and added into a sterile flask containing glass beads. The mycelia were broken by vortexing in the presence of glass beads, and 2 ml of this solution was inoculated into the sterile flasks containing different solid substrates followed by incubation at RT for five days. After the incubation period, Tris buffer (0.05 M) was added in each flask, shaken and filtered through Whatman filter paper. The filtrate was collected, and enzyme activity was performed at RT, as mentioned before.

Examination of plant growth-promoting ability of fungal isolate GU-S

Fungal isolate GU-S was examined for the properties of plant growth promotion, which is characterised by production of indoleacetic acid (IAA), hydrogen cyanide (HCN), siderophores, phosphatase enzyme (Ahmad et al. 2008). All the tests were carried out by incubating the isolate GU-S for 7–10 days depending upon the growth of the culture. Inoculum for the tests was prepared similarly, as mentioned for SSF.

Use of asparaginase in plant growth promotion (PGP)

Pisum sativum was chosen for the pot level experiment for evaluation of plant growth promotion as its seeds are protein-rich (Mamontova et al. 2018). Proper care was taken before using the seeds for the experiment (Dastager et al. 2010). Growth of the seeds was examined in five replicates under two conditions; test and control. For the test, the culture filtrate of isolate GU-S was used, which was grown in MSM containing substrate L-asparagine and incubated for 72 h under aeration. For the control, the culture filtrate containing MSM without L-asparagine but with glucose was used maintaining uniform growth conditions as that of the test flask. An equal amount of autoclaved soil was added in all pots for the growth of *Pisum sativum* plants. Germinating seeds were dipped in test and control culture filtrates separately for 2 h at RT followed by sowing them into different pots containing sterile soil. Plant growth was monitored for twenty days. Hence the final measurements of shoot and root length were done twenty days after sowing. The whole experiment was repeated to confirm the effect statistically.

Statistical analysis

For the plant growth experiment, the root and shoot length of test and control seedlings were analyzed statistically to determine whether the differences observed were significant or not. A two-sample t-test was performed separately on root and shoot length of test and control using MS Excel 2007.

Results

Identification of fungal isolates

Fungal isolate GU-S clustered along with *Peroneutypa* species in the Neighbour Joining Tree and hence was identified as a species under *Peroneutypa* (**Fig. S1**). Isolate GU-G clustered along with *Lecanicillium* species and identified as a species under *Lecanicillium* (**Fig. S2**). The former belong to Diatrypaceae (Xylariales, Sordariomycetes) and the latter belongs to Cordycipitaceae, (Hypocreales, Sordariomycetes). The sequences have been deposited in GenBank with accession numbers MK614937 and MK614938, respectively.

Enzyme estimation

Asparaginase activity displayed by the *Peroneutypa* sp. GU-S and *Lecanicillium* sp. GU-G in the broth was 1.09±0.04 and 0.70±0.13 U, respectively.

Effect of aeration on enzyme production

Peroneutypa sp. GU-S showed higher enzyme production than *Lecanicillium* sp. GU-G at both temperatures tested for activity (**Fig. 1**). In *Peroneutypa* sp. GU-S, production of the enzyme that was active at RT increased with aeration. In contrast, the enzyme that was active at 37 °C did not show any difference between the presence or absence of aeration on the production of the enzyme.

Effect of different conditions on enzyme activity

Temperature

Both the fungal isolates were grown at RT, but the enzyme produced by them was active at RT as well as 37 °C. Moreover, the enzymes produced by both showed better activity at 37 °C than at RT (**Fig. 1**).

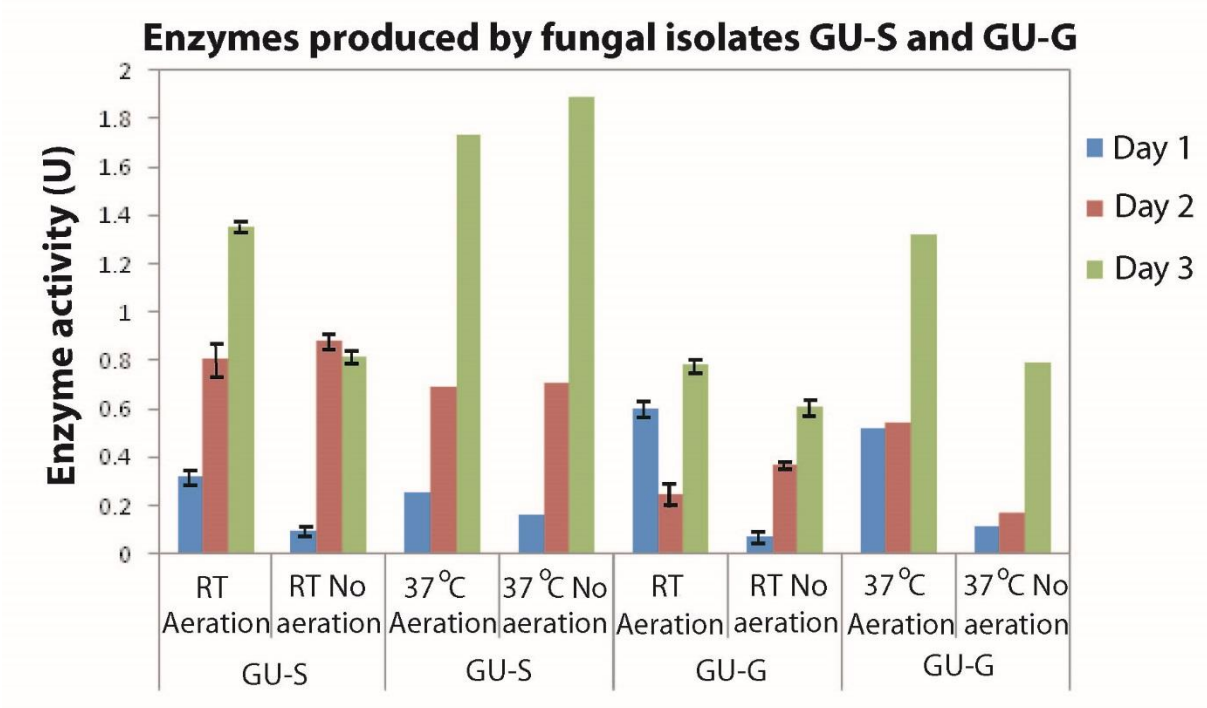


Figure 1. Effect of aeration on production of L-asparaginase by the two fungal isolates and the effect of temperature on enzyme activity of the same. 'RT' and '37 °C' on the X-axis refer to the temperatures at which enzyme activity was examined, and 'Aeration' and 'No aeration' refer to the shaker and static conditions, respectively, at which enzymes were produced.

pH (4, 5, 6, 7, 8, 9 and 10)
Higher enzyme activity was observed at pH 6 and 10 for *Lecanicillium* sp. GU-G and pH 8 for *Peroneutypa* sp. GU-S (**Fig. 2**). The enzyme activity was least at pH 4 for both the isolates.

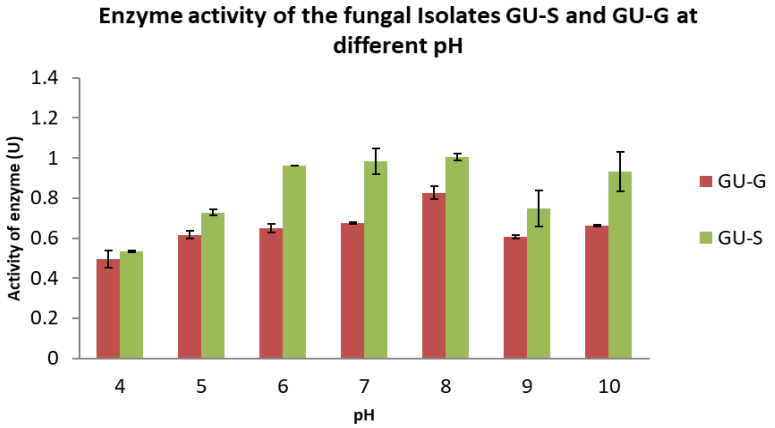
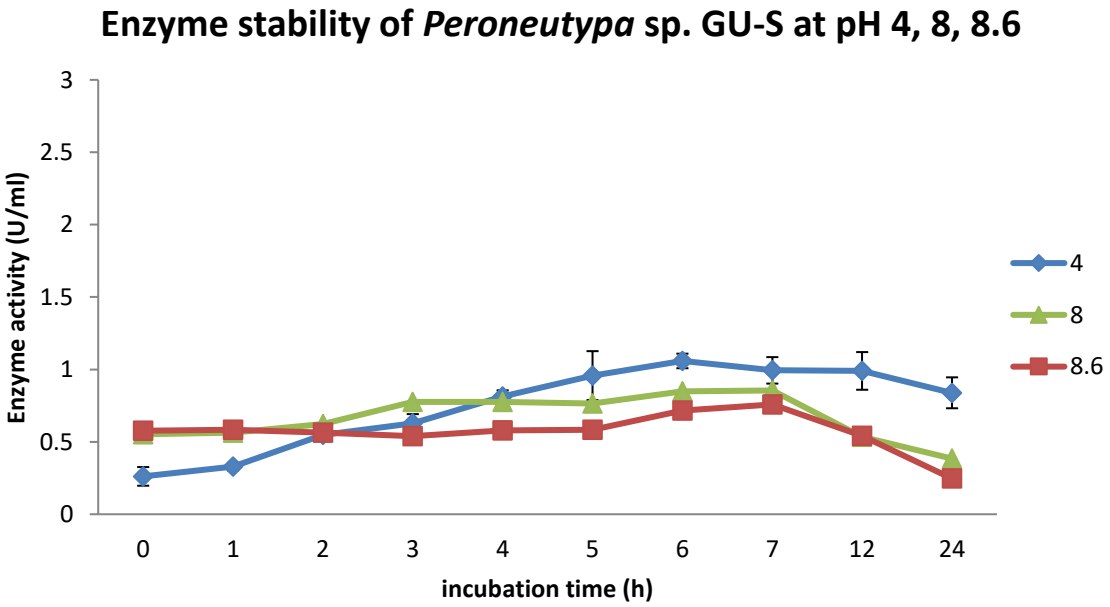


Figure 2. Effect of pH on enzyme activity of the two fungal isolates

Enzyme stability at different pH
The enzymes produced by both the fungal isolates were more stable at pH 4 than the rest of the pH checked (**Fig. 3**). The enzyme activity increased by 1.4 fold in the first 3 hours of incubation at pH 8 and 8.6 and by 2.4 fold at pH 4. At the latter pH, the activity increased by 4 fold by 6 h and remained stable till 24 h, i.e. till the end of the incubation period.



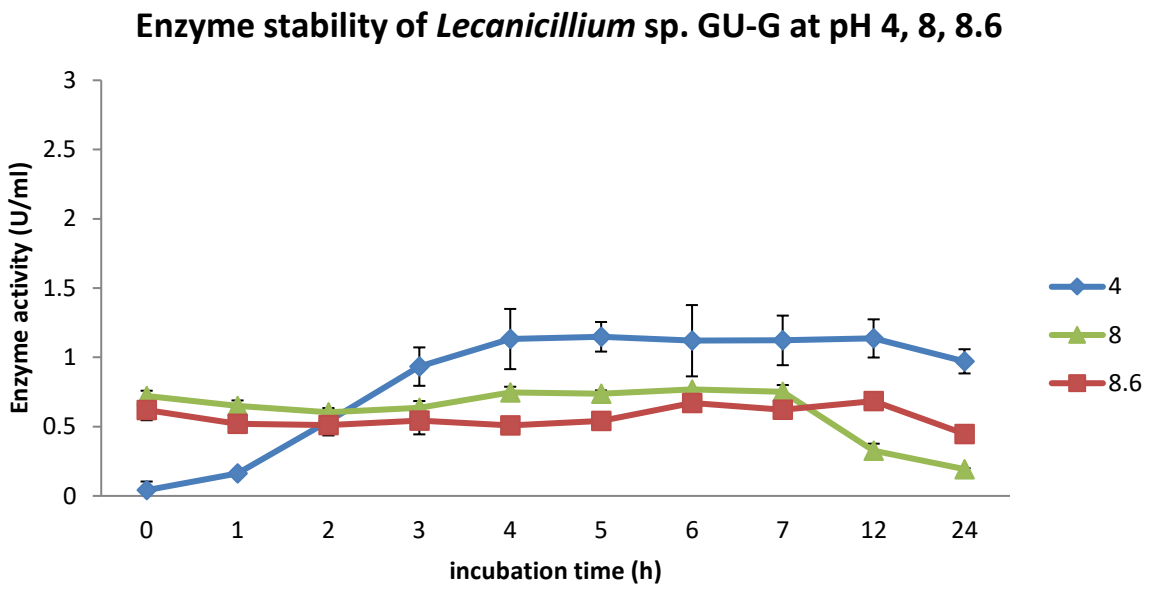


Figure 3. Enzyme stability of fungal isolates at different pH

Determination of inexpensive sources for growth of the fungi

The flasks containing soya and grass showed good growth, whereas the flasks containing sugarcane bagasse and wheat bran did not show any significant growth. The enzyme produced in the presence of soya and grass as determined by the enzyme assay was 3.516 and 0.146 U, respectively (**Fig. 4**).

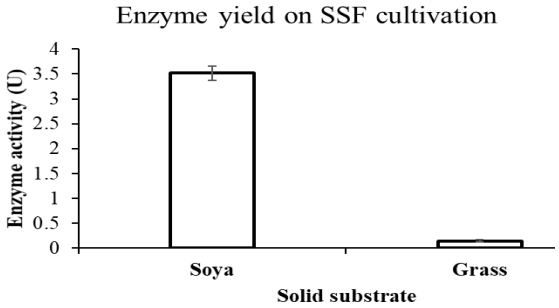


Figure 4. Enzyme activity of GU-S with soya and grass as a substrate for SSF.

Examination of plant growth-promoting ability of Isolate GU-S

Peroneutypa sp. GU-S that was used for the plant growth experiment displayed no positive reaction towards tests for indoleacetic acid, hydrogen cyanide and siderophore production, whereas the phosphatase production test was positive. Zone of decolourization (5 mm from the edge of the colony) was observed on Pikovskaya's agar after seven days of incubation.

Use of asparaginase in plant growth promotion (PGP)

The test seedlings showed a better and consistent growth as compared to the control seedlings (**Figs. 5 and S3)**. The seedlings in control (uninoculated) pot showed varied dimensions (irregular pattern represented by high standard deviation) as compared to the test. The shoot and the root length were 1.2 and 2.2 times more in the test than in control within twenty days of incubation. This was confirmed statistically by t-test wherein $t_{stat}>t_{crit}$ with p=0.0166 for shoot length ($t_{statistic}$=2.29, $t_{critical}$=1.73) and p=0.0375 for root length ($t_{statistic}$=1.88, $t_{critical}$=1.73) with degrees of freedom as 22.

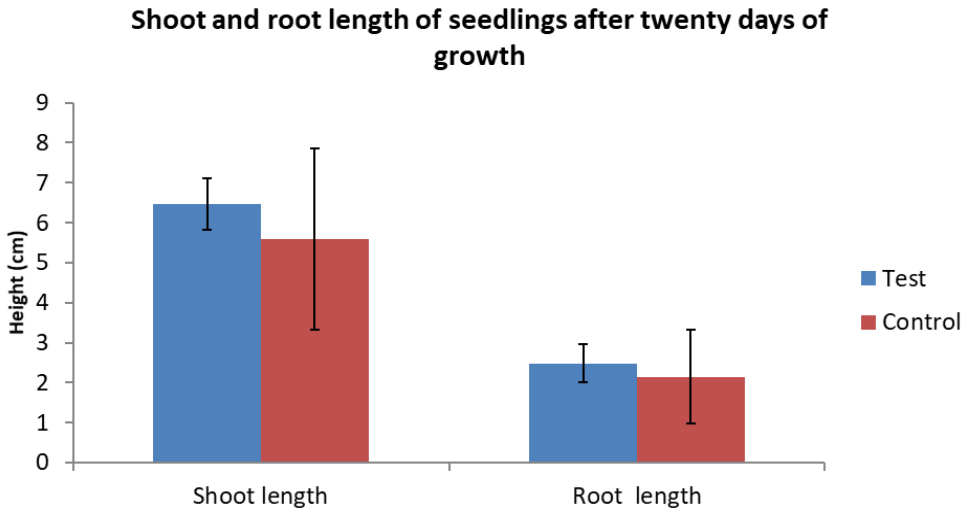


Figure 5. Differences in root length and height of the plants under test (seeds+MSM with crude enzyme) and control (seeds+MSM only) conditions.

Discussion

In this study, L-asparaginase enzyme was produced in laboratory conditions by two fungal isolates obtained from mangrove detritus. To date, there has been only one study on fungal asparaginase from mangrove ecosystem (Gupta et al. 2009). Most commonly occurring fungi such as *Aspergillus*, *Alternaria*, *Penicillium* and some pathogenic fungi (such as *Helminthosporium*, *Fusarium*) produce L-asparaginase enzyme (Gupta et al. 2009). In this study, we obtained two fungal isolates which belong to *Peroneutypa* sp. and *Lecanicillium* sp.; the asparaginase of which has not been reported earlier. The former is known as an endophyte while the latter is an entomopathogen. Entomopathogenic fungi protect the plants by infecting the pests and releasing the organic nitrogen from their cadavers into the soil to serve as nutrition to the plants (Humber 2008). Some of these fungi survive inside the plants as endophytes (Tall and Meyling 2018). Several endophytic fungi promote plant growth by acting as growth stimulator or biocontrol agent (Hermosa et al. 2012, Saba et al. 2012, Chen et al. 2016). The biocontrol and growth stimulator characteristics of entomopathogenic and endophytic fungi also confer resistance to plants against abiotic and nutrition stress (Hermosa et al. 2012, Raya-Díaz et al. 2017). In the present study, the effect of the enzyme on plant growth promotion was examined rather than the effect of the culture. Hence studying the entomopathogenic and endophytic nature of the cultures that confer stress resistance to plants was beyond the scope of this study. The production of L-asparaginase from entomopathogenic and mycoparasitic fungi recovered from mangrove detritus has not been known till date to the best of our knowledge.

The mangrove detritus can be a rich source of fungi from plant origin as well as from insect origin, as fungi are decomposers of organic material from both the sources (Humber 2008). The autochthonous organisms are exposed to a wide variety of climatic conditions, i.e. changes in temperature and salinity as well as to terrigenous organic and inorganic pollution (Ramanathan et al. 2008). Therefore such isolates are quite sturdy, and the enzymes produced by these could probably be stable at a wide range of conditions. L-asparaginase from organisms isolated from mangrove habitats would thus provide an added advantage.

Enzymes are usually sensitive to a pH fluctuation, and their activity is hampered at wide pH ranges. pH is an important physical parameter besides temperature that is known to influence the enzyme production, activity and growth of an organism itself (Wakil and Adelegan 2015). For *Fusarium oxysporum*, Yadav et al. (2014) has shown maximum pH activity range to be of pH 5. In the present study, the enzyme activity of the fungal isolate GU-G was found to be almost constant at pH range 6, 7, 8 and 10. Even isolate GU-S showed almost similar activity at most of the pH except 8 wherein the activity increased. Hence, enzyme stability was studied at this pH. Stability was also checked at pH 4 where the activity was least and pH 8.6, which was the pH used in the enzyme assay. Enzymes from both the fungal isolates were stable at the wide range of pH supporting the versatile nature of mangrove derived isolates.

In the present study, the fungal isolates were grown at RT, and thus the temperature for enzyme production was RT, but it showed better activity at 37 °C. The activity of L-asparaginase has been checked in most of the studies at a temperature range of 30 to 45 °C (Batool et al. 2016). Enzyme activity in the present study was checked at only two different temperatures viz., RT and 37 °C, to examine if the enzyme can serve the dual purpose of plant growth promotion and anti-tumour agent in humans. Shatavari, which contains a large amount of L-asparagine, was used to induce L-asparaginase production by *Peroneutypa* sp. GU-S during SSF. Growth and activity of this isolate on only soya and grass among all the substrates studied needs further investigation. Solid-state fermentation is a very efficient technique for L-asparaginase production by fungi because the yield of the product is much more than submerged technique (El-Bessoumy et al. 2004). High-level production of L-asparaginase from a strain of *Aspergillus niger* by SSF using agro-wastes has been achieved earlier by Mishra (2006).

The ability of the isolate GU-S to grow by SSF and produce the enzyme gives an advantage for commercial production of enzyme and its application as biofertilizer in future. The plant growth experiment consistently resulted in the production of better seedlings in test pot than in control, thus pointing towards the use of L-asparaginase in plant growth promotion. The seeds inoculated along with crude enzyme, i.e. MSM culture broth clearly showed a steady growth pattern, which indicated that L-asparaginase, if provided externally, can positively influence plant growth. We believe that the uniform growth in all test seedlings was due to degradation of L-asparagine, present abundantly in the seeds, by L-asparaginase. L-asparagine degradation would provide ammonia to the plant, and since it is readily utilizable source of nitrogen, it ultimately promoted the steady growth of the seedlings. The positive effect of test culture broth on plant growth was confirmed statistically by t-test.

The isolate GU-S whose culture filtrate showed a positive effect on plant growth did not produce any plant growth-promoting factor other than phosphatase. Moreover, this phosphatase was produced after seven days of incubation in the culture medium. The crude enzyme extract used in the plant growth experiment was obtained after harvesting the culture filtrate of GU-S on three days of incubation. The L-asparaginase enzyme was induced within the first day of incubation in the culture broth. Hence the chances of phosphatase contributing to the plant growth promotion of the seedlings in the experiment are less likely to occur because the chances of phosphatase being present in the MSM broth were meagre.

In conclusion, the detritus-derived fungi from mangrove ecosystem are potential sources of L-asparaginase. The enzyme derived from mangrove source bears stability over wide pH range and can be exploited for its use in plant growth promotion. Its activity at 37 °C may facilitate its use in biomedical application also. Mycoparasitic and endophytic fungi can thus produce L-asparaginase at par with most commonly occurring fungi. L-asparaginase may be considered

as plant growth-promoting factor owing to its positive effect on the growth of *Pisum sativum* seedlings.

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Author contributions
VSD designed the experiments and carried out statistical analysis. KGK carried out the experiments. Both VSD and KGK prepared the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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