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# Effect Of *Trichoderma Viride* On Growth, Yield And 4-Hydroxyisoleucine Content Of *Trigonella Foenum-Graecum* (Fenugreek)

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**ABSTRACT:** *Trigonella foenum- graecum* (fenugreek) is a leguminous plant with poor nitrogen fixing ability. The current paper deals with the effects of *T.viride* on growth, yield and 4-hydroxyisoleucine content in fenugreek. 4-hydroxyisoleucine is an antidiabetic compound present only in plants particularly in fenugreek. Treatment of fenugreek seeds with *T.viride* enhanced the growth, yield and 4-hydroxyisoleucine content significantly compared to control. Seeds were treated with *T.viride* suspension ($10^7$spores/seed) and untreated seeds (1 ml distilled water/ seed) served as control were sown in pots filled with potting soil and carried out various experiments after the growth of plants. Several growth parameters like root length and lateral root expansion, shoot length, yield, chlorophyll and nitrogen were observed and estimated. 4-hydroxyisoleucine content in fenugreek seeds was estimated by ninhydrin method. A significant increase of 25 % in root length and 128 % in lateral root expansion and 50 % in shoot length was observed in treated plants over control. The total yield increased by 95 % in treated. The total chlorophyll content exceeded by 21.86 % and total plant nitrogen by 121.73 %. An increase of 27 % of 4-hydroxyisoleucine content is observed in treated fenugreek plants over control.

***Keywords*:** *Trichoderma Viride, Trichoderma Viride, Trigonella Foenum-Graecum*

## INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) known as *methi* in India is used as a condiment and flavoring agent in Indian cuisine. It is a semi arid crop, cultivated worldwide primarily for its seeds, leaves and only secondarily for forage. Fenugreek is known to have originated from South-East Europe and West Asia (Ecocrop, 2017, Alaoui, 2005). It belongs to the subfamily *Papilionaceae* and family *Leguminaceae*.

Fenugreek plants are annual herbaceous, erect plants growing to a height of 40-80 cm (Ecocrop, 2017). It is tap rooted with finger like projections (Moradi kor and Moradi, 2013) and trifoliate leaves (Srinivasan, 2006; Basu, 2006). Flowers are triangular (Flammang *et al*., 2004) and pale yellow or whitish in color (Ecocrop, 2017). Flowers produce cylindrical pods with curved tips (Figure 1). Seeds are light brown with strong characteristic and spicy odour (Ecocrop, 2017, Alaoui, 2005). Fenugreek leaves and inflorescence are regulated by temperature (McCormick *et al*., 2006).

The germination time for fenugreek is 4-6 days and the percentage of germination is 90-95%. During the process of germination, the nutritional content of the crop is enhanced (Pandey and Awasthi, 2015). Germinated fenugreek seeds have higher antioxidant content and enhanced antidiabetic activity than its boiled counterpart (Naidu and Shyamala, 2011).

Fenugreek is the oldest cultivated spice crop known to Indian diet 3000 years ago, for its qexceptional nutritional profile. Ancient Chinese medicine regarded fenugreek as a remedy for edema and feebleness of legs (Yoshikawa *et al*., 2000). In India, fenugreek is mostly used as a seasoning agent in cookery (Betty, 2008). Fenugreek seeds are known for their hypocholestrolemic, antidiabetic, anticancerous and several other activities. These beneficial effects are mainly attributed to intrinsic dietary fiber constituent having nutraceutical value (Srinivasan, 2006).

India is a major producer and exporter of fenugreek. Fenugreek is cultivated during the Rabi and Kharif seasons in India. Rajasthan considered as fenugreek bowl of India produces 80 % of total fenugreek and is grown as cash crop by farmers.

Fenugreek seeds add to the nutritive value and hence are used as a spice in vegetarian diet. Fenugreek seeds are rich in carbohydrates, protein, fats and Vitamin A and have high calorific value of 323 Kcal per 100 gm. They are also rich in minerals like iron and copper. The percentages of the respective components are given in appendix A.

Oral intake of fenugreek stimulates insulin production resulting in hypoglycemic effect (Roberts, 2011). Reported studies have brought to light that optimal consumption of fenugreek lowers cholesterol and triglyceride proportions in blood (Srivatsava *et al*., 2012).

Success of fenugreek in field is subject to several climatic and environmental conditions. Suitable management techniques like sowing time, fertilization, irrigation etc. leads to improved crop production. Especially sowing time is most important under dry land conditions in continental climate.

Sowing time is the determining factor in the yield of spring crops affecting longevity of growing period (Nandre *et al*., 2011). Delay in sowing time decreases duration of growing period. Low temperature and excess water in the seedbed are main obstacles in early sowing in continental climate areas. The optimum sowing date results in efficient use of light, time, temperature, precipitation and other factors (Moosavi, 2014). Cutting management and pinching practices also influence the growth and yield of fenugreek (Baboo, 1997). Apical bud pinching helps in production of side shoots resulting in increased photosynthetic activity and accumulation of more photosynthates resulting in increased seed size and yield (Lakshmi *et al*., 2015).

Germination is an intense physiological process under the control of environmental factors such as temperature, water and light (Shaban, 2013). Suitable temperature and irrigation are necessary for initiation of enzyme activation and rapid imbibition and germination (Baskin and Baskin, 2001).

Naturally growing legumes in arid and semi-arid regions are subjected to severe environmental challenges. Germination generally increases with increasing temperature until optimum temperature. The optimum temperature for germination changes between 10 and 20 °C for cool season species like fenugreek (Baskin and Baskin, 2001). Lower temperature stimulates seed germination of some plant species under lower water stress condition (Shaban, 2013).

Approximately, 80 % of total earth's atmosphere is composed of nitrogen gas ($N_2$), which cannot be utilized by most of the living organisms. Nitrogen is regarded as an essential nutrient for plants, which is usually deficient in soils, leading to reduced agricultural yields throughout the globe. Soil microorganisms act as biogeochemical agents in converting the complex macromolecules into simple reusable forms. This process is called mineralization (Pelczar *et al*., 1996).

The process of nitrogen fixation with the help of nitrogen fixing microbes involves conversion of inert nitrogen gas into organic useful ammonia. (Sorensen and Sessitsch, 2007). Some bacteria are capable of fixing nitrogen symbiotically (Geurts and Bisseling, 2002).

Fenugreek is a useful legume crop for short-term rotation (Tunkturk *et al*., 2011). Although it is reported that fenugreek may fix 48 % of its total $N_2$ during growing season (Poi *et al*., 1991), it is a weak nitrogen fixer, owing to the low seed mass, low plant biomass and inefficient expansion of lateral roots. The major traits responsible for nitrogen uptake from soil are lateral root expansion and early biomass, which are related to seed mass (Dayoub and Elana *et al*., 2017).

Legumes have slow root depth penetration (Bellostas *et al*., 2003; Corre-Hellou *et al*., 2007; Thorup-Kristensen, 2001). Moreover soil nitrogen uptake was correlated to lateral root expansion rate. Branched roots explore the soil in a better way during early stages. The soil nitrogen availability does not modify the root depth penetration (Corre-Hellou and Crozat, 2005) but is known to modify the lateral root expansion.

Legumes are known to acquire low nitrogen during their early crop lifecycle. This can be attributed to the fact that nodule formation and infection by symbiotic bacteria require a significant period of time for development. During initial growth stages, legumes access three distinct sources - soil nitrogen, atmospheric nitrogen (to a lower extent) and seed reserve (Dayoub *et al*., 2017).

The compound 4-hydroxy isoleucine was first isolated and identified in fenugreek seeds (Fowden *et al*., 1973). This branched-chain, unusual amino acid is present exclusively in plants notably in fenugreek seeds (0.015 % - 0.4 %) but never in mammalian tissues (Mehrafarin *et al*., 2010). It is a non-proteinogenic amino acid (Sauvaire *et al*., 1991) synthesized from isoleucine. The property of regulation of pancreatic insulin secretion makes it an effective antidiabetic molecule (Sauvaire *et al*., 1998; Fowden *et al*., 1973). These insulinotropic properties of 4-OHIleu make it a potent molecule in treating diabetes unlike sulfonylureas that induce hypoglycemia (Fuller *et al.*, 2015). It constitutes 80 % of total free amino acid content of fenugreek. 4-OHIleu reduces insulin resistance and functions as an insulin secratagogue at elevated glucose levels, 8.3-16.7 millimolar (mM) range (Broca *et al*., 2000b).

The compound 4-OHIleu not only plays a prominent role in insulin secretion but also regulates glucose metabolism thereby reducing body fat (Sharma and Raghuram, 1990). This amino acid is strictly glucose dependent. The glucose concentration in blood is directly proportional to insulin stimulating effect exhibited by 4-OHIleu. It serves as an adaptogen, by helping in regulating insulin needs (Sauvaire *et al*., 1991). The amino acid 4-OHIleu exhibits nutrient partitioning effect, which means it prefers to shuttle nutrients to muscle cells over fat cells (Sauvaire *et al*., 1991).

Traditional agricultural practices are being affected to a large extent by several problems like diseases, pests, excessive use of chemical pesticides and pollution. Thus there is a need for biocontrol agents, which help in alleviating some of these problems. Biological control is defined as the interference of specialized microbes against pests and plant pathogens in an ecofriendly alternative to overcoming the practice of chemical fertilizers (Harman *et al*., 2004).

*Trichoderma* species is omnipresent in varied habitats (Harman *et al*., 2004), is a low cost versatile biocontrol agent and also good fertility promoter establishing itself in different pathosystems. Their potential as biocontrol agents was established since1930's. In addition to plant growth enhancement *Trichoderma* boosts seed vigor (Shoresh *et al.*, 2010), improves efficacy of plant nitrogen uptake (Harman, 2011) and solubilizes micronutrients like iron, copper, manganese and zinc in soil. They produce enzymes and metabolites that modify the root architecture resulting in adequate nutrient uptake. Preferred *Trichoderma* strains upgrade nutrient uptake and are frequently employed as seed treatments, which bring on improvements in plant growth and quality in distant future. Employment of *Trichoderma* as a biocontrol agent greatly influences the seed germination and seedling vigor (Celar and Valic, 2005). Early seed-*Trichoderma* species interactions provide insights into plant performance on a long-term basis.

Various studies have shown that *Trichoderma* species counteract, parasitize, or even turn out other fungi with their competency to reduce plant attacks by the pathogens (Monte, 2001).

Competition through rhizosphere know-how is of paramount importance, as the biocontrol agent thrives for space and nutrients if it is incompetent in the rhizosphere. *Trichoderma* are efficient competitors for nutrition and living space (Hjeljord *et al*., 2000). They modify rhizospheric soil by acidification to rule out the pathogen development (Benitez *et al*., 2004). Simultaneous growth of *Trichoderma* species is established in roots of the treated plant with direct application to soil or as seed treatments. (Harman, 2000, Howell *et al*., 2000) Starvation is another important cause of microbial death, so competition for limited nutrients makes it an effective bio control agent. Iron uptake is imperative for fungi and under iron deprivation they produce ferric-iron specific chelators, called siderophores. These siderophores are efficient iron chelators which inhibit other fungi (Benitez *et al*., 2004). *Trichoderma* protein production plays a vital part in colonizing roots which is known to be pivotal in competing with various root colonizers and a few help in establishing symbiotic affinity towards host plants (Samolski *et al*., 2012). Root colonization by rhizospheric competent *Trichoderma* species result in marked development of root and shoot organization and the yield.

*Trichoderma* produces several antibiotics and secondary compounds that play a vital role in biocontrol. Mycoparasitism is a major antagonistic mechanism of *Trichoderma* (Che'rif and Benhamou, 1990). It has the capability to increase induced resistance in host plants when pretreated (Harman, 2004) against pathogens (Yedida *et al*., 1999). Recent studies revealed that *Trichoderma* acts as an endophytic symbiont in arboraceous plants (Chaverri and Gazis, 2011). This union helps in nitrogen uptake, increased photosynthetic rate giving rise to greater outputs (Chaverri and Samuels, 2013).

Keeping the above points in view the current paper revolves around the hypothesis that enhanced nitrogen uptake facilitated by *T.viride* might enhance the 4-hydroxy isoleucine content of fenugreek.

## MATERIALS AND METHODS

### Maintenance of fungal culture

Fungal culture *Trichoderma viride* TV10 strain was procured from PDBC Bangalore. The culture was maintained and sub cultured on Sabouraud's dextrose agar (SDA) medium. Spore suspension of *T. viride* TV 10 was prepared in distilled water containing $10^7$ spores/ml.

### Seed procurement and treatment

Fenugreek seeds were procured from local market. Seeds were incubated for 24 hours in the fungal spore suspension (1 seed/1 ml of suspension). Seeds incubated in urea suspension (1seed/1ml of suspension) served as positive control and those incubated in distilled water served as control (1 seed/1 ml of distilled water). After 24 hours, they were sowed in 13inch pots, which were filled with 3 kilograms of potting soil.

### Determination of growth parameters

Growth parameters like root and shoot lengths, chlorophyll content, total nitrogen content and 4-hydroxyisoleucine content and yield (pod length, number and size of seeds per pod) were observed.

### Root and shoot lengths

Plants were uprooted periodically for every 15 days until harvest from the date after sowing (DAS) and their root length, lateral root expansion and shoot lengths (in cm) were noted.

### Yield (Pod length, seed size and their number per pod)

At the time of harvest (90DAS), pods were removed, dried in the sun and their lengths were measured. Seeds were removed from the pods and dried under the temperature of 35-45 °C until the seed weight was stable. Yield (seed size and their number per pod) is calculated in terms of number of seeds harvested per each seed sown.

### Estimation of chlorophyll content

Fresh leaves (30 days old) were collected and chlorophyll content was estimated by acetone method (Arnon, 1949). Fresh leaf material (0.2 g) was placed in a test tube and ground with 80 % acetone using mortar and pestle. The chlorophyll extracted in acetone was collected and filtered using Whatman no.1 filter paper. The homogenate was washed with 5 ml of 80 % acetone for at least twice or thrice each time. The resulting volume of obtained filtrate was made to 25ml using 80 % acetone. The intensity of chlorophyll a, b and total chlorophyll were estimated by examining optical densities at 663 and 645 nm.

The extent of chlorophyll present in the obtained solution was calculated in terms of mg chlorophyll per g tissue by applying the following equations

Equation (1) mgchlorophylla / gtissue = $12.7(A663) - 2.69(A645) \times \frac{V}{1000 \times W}$

Equation (2) mgchlorophyllb / gtissue = $22.9(A645) - 4.68(A663) \times \frac{V}{1000 \times W}$

Equation (3) mgtotalchlorophyll / gtissue = $20.2(A645) + 8.02(A663) \times \frac{V}{1000 \times W}$

Where

A = absorbance at specific wavelengths
V = final volume of chlorophyll extract
W = fresh weight of tissue extracted

### Total nitrogen estimation

Seeds were treated and sown in three pots namely control, positive control and treated as discussed under 3.2. The total plant nitrogen content was estimated by Micro-Kjeldahl method (Kjeldahl, 1883). This method is carried out in three steps:

In the first step (digestion) 100 mg of plant sample is digested with 2 ml of sulphuric acid in a micro Kjeldahl digestion tube in the presence of a pinch of mixed catalyst which is a combination of 3.5 g of copper sulphate, 0.5 g of Selenium dioxide and 96 g of sodium sulphate to increase the boiling point of the medium. Digestion is carried out for about six hours until all the nitrogen has been converted to ammonia. The solution is cooled and the volume is made to 50 ml with sulphuric acid.

In the second step (distillation) 5 ml of the digested sample is transferred to steam distillation apparatus to which 5 ml of 40 % sodium hydroxide is added and is boiled to generate steam, which was previously cleansed by carrying a blank distillation. Mixed indicator containing five parts of 0.1 % bromocressol green with one part of 0.1 % methyl red solution along with 10 ml of 2 % boric acid is placed in the receiver flask which is arranged in such a way that the end of the condenser outlet was just lowered beneath the surface of the receiving fluid. 10 to 15 ml of distillate is collected in the receiving flask.

In the final step titration the remnants of the receiving flask were titrated with 0.01 N sulphuric acid until the solution is brought back to its original pink color. The color change is observed from bluish green to pink. The volume of sulphuric acid run down was noted.

The amount of nitrogen in the sample is calculated as follows

Equation (4) $1\ ml\ of\ 0.01\ N\ H_2SO_4 = 0.14\ mg\ of\ N_2$

$x\ ml\ (titre\ value) of\ H_2SO_4 = 0.14x\ mg\ of\ N_2$ (present in 5 ml of digested sample)

$50\ ml\ of\ H_2SO_4 = \frac{0.14x \times 50}{5} mg\ of\ N_2$

**Estimation of 4-hydroxyisoleucine content**

Free amino acids were estimated by Moore and Stein method, developed in 1948. Five hundred milligrams of ground fenugreek seeds were taken and to it 10 ml of 80 % ethanol was added and filtered. This extraction was repeated twice and pooled. The volume of extract was reduced by evaporation and free amino acids were estimated by ninhydrin method. Leucine was used as a standard. 4-hydoxyisoleucine was estimated by multiplying the amount of free amino acid with 0.80.

## RESULTS

**Fungal culture**

*Trichoderma viride* shows speedy growth on Sabouraud's dextrose agar. Whitish mycelial progress (growth) was observed subsequently in three days. Complete sporulation was seen after five days, comprising dark green spores. The conidiophore branching pattern was terverticillate, short phialides and globose to ellipsoidal conidia (Rifai, 1969) and bluish green to dark green spores were observed. The stained image of TV 10 strain is shown (Figure 1).

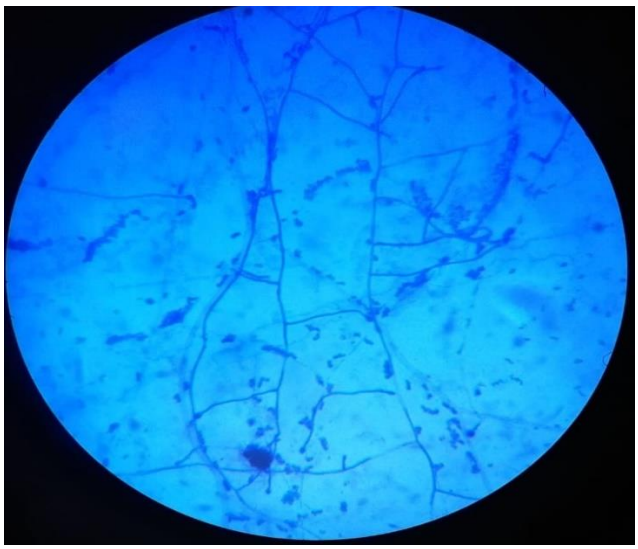


Fig 1: Stained image of *T.viride* TV 10 under oil immersion lens

**Effect of *T.viride* treatment on growth parameters**

**Root and shoot lengths**

Lengths (segments) of roots and shoots were measured and found to be higher in treated plants (Table 1 &Figure 2). An average increase of 25% in the root segment was observed in treated plants (Figure 3). Lateral root development was found to be more profound and branched in treated plants over control (Figure 4 & 5). There is 128 % increase in the bulk of lateral roots in treated plants (Figure 8). A striking increase of 50% in shoot segment was inspected in treated when compared to control plants (Figure 3).

Table 1: Effect of *T. viride* on root and shoot lengths of fenugreek

| Sample | Root length (in cm) | Shoot length (in cm) |
|---|---|---|
| Control | 5.5 ± 1.31 | 15.8 ± 1.61 |
| Treated | 6.9 ± 0.23 | 23.72 ± 1.30 |

Values represent mean (n=100) shoot and root lengths ± SE; ANOVA *p* value<0.005


Fig 2: Root and shoots of control and treated fenugreek.

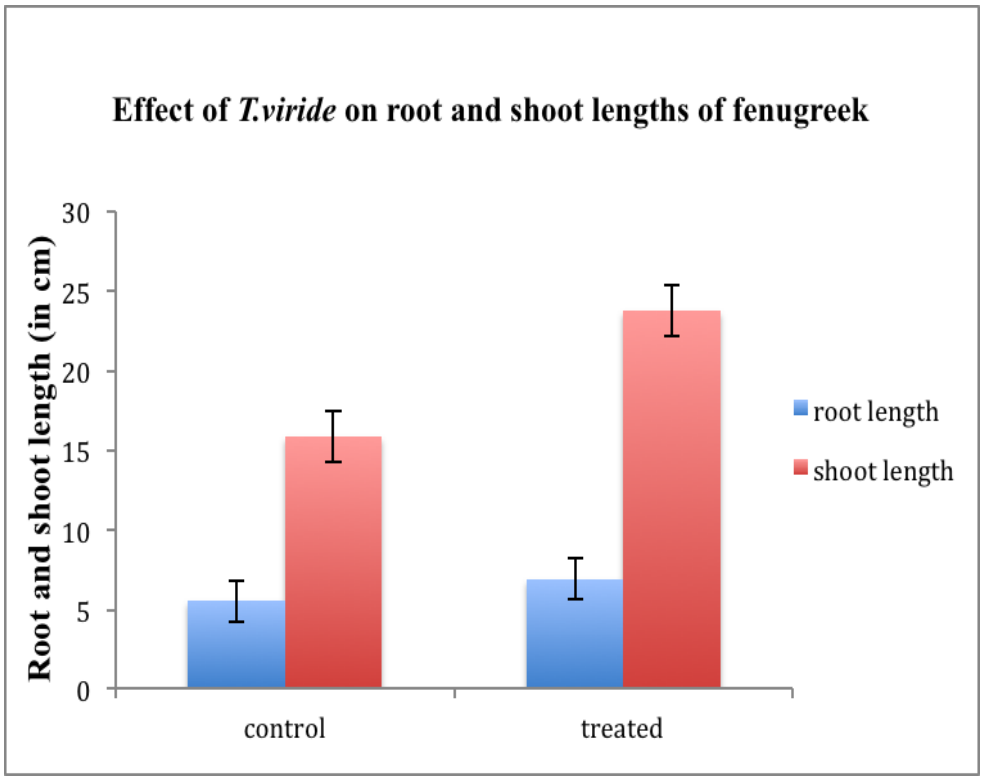



Fig 3: Effect of *T.viride* on root and shoot lengths of fenugreek

Table 2: Effect of *T.viride* on lateral root expansion of fenugreek

| Sample | Number of lateral roots |
|---|---|
| Control | 11.25 ± 0.97 |
| Treated | 25.75 ± 1.8 |

Values represent mean (n=100) number of lateral roots ± SE; ANOVA *p* value < 0.005

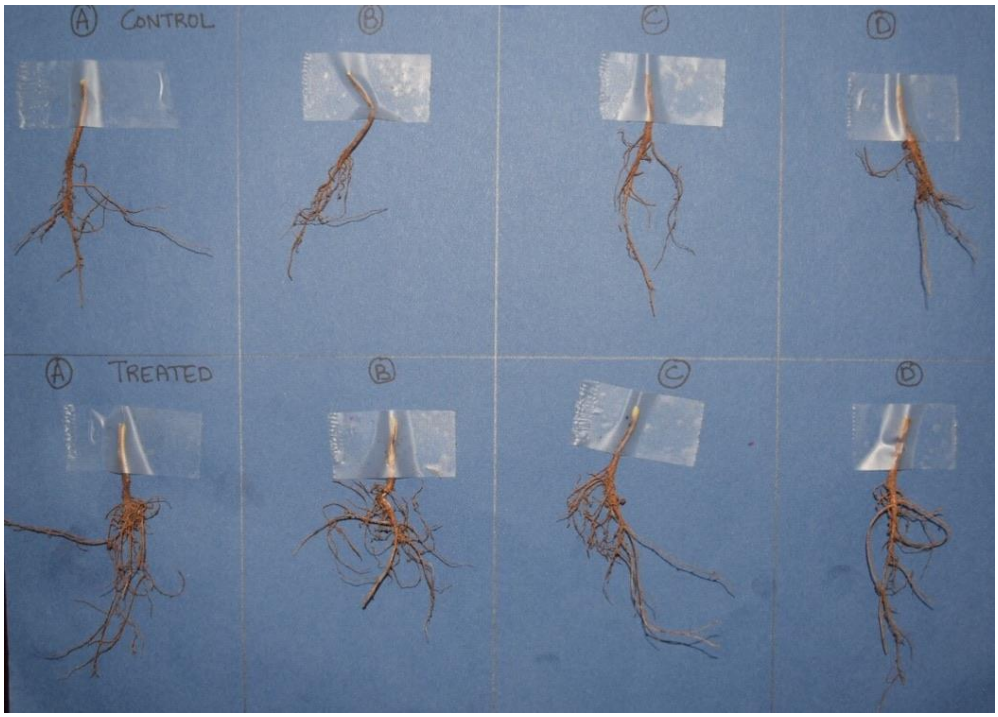
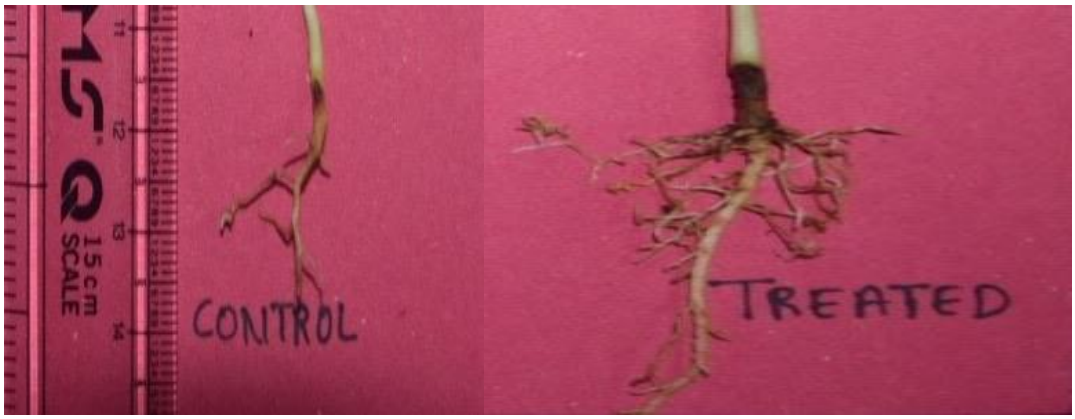



Fig 4: Lateral roots of control and treated fenugreek plants




Control Treated

Fig 5: Lateral root expansion of control and treated plants

Fig 6: Nodule formed in one of the treated plants

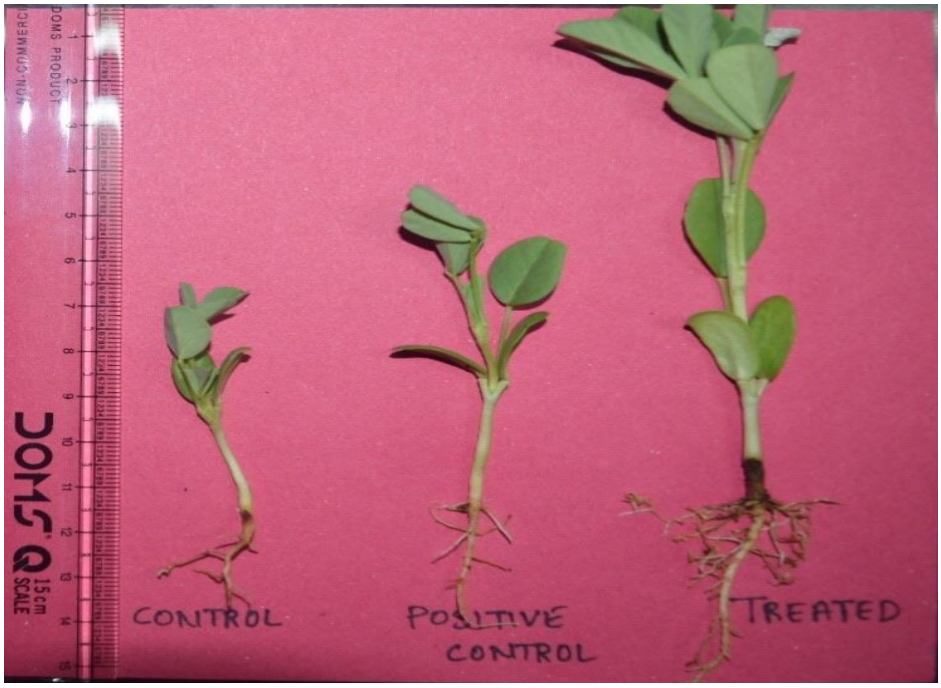



Fig 7: Root and shoot lengths of control, positive control (NPK) and treated plants

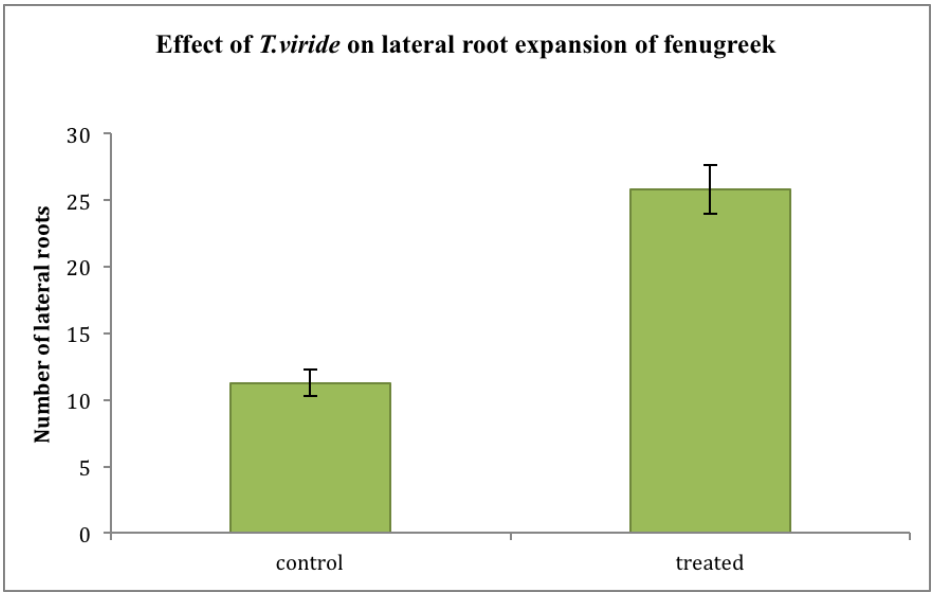



Fig 8: Effect of *T.viride* on lateral root expansion of fenugreek

**Yield (Pod length, seed size and seed number per pod)**

Pods and seeds of treated plants were large and big (Table 3 & Figure 9, 10 & 12) and more in number over control. A 54 % increase was observed in the pod length in treated plants (Figure 11). The average yield of treated plants obtained was 5 seeds per each seed sown and 3 seeds for control. The yield (number of seeds per pod) increased by 95 % (Table 4 &Figure 13).

Table 3: Effect of *T.viride* on pod length of fenugreek

| Sample | Pod length (in cm) |
|---|---|
| Control | 7.44 ± 0.38 |
| Treated | 8.44 ± 0.38 |

Values represent mean (n=100) pod length ± SE; ANOVA *p* value < 0.005


Fig 9: Fenugreek pods of control and treated plants


Fig 10: Fenugreek pods of control and treated plants

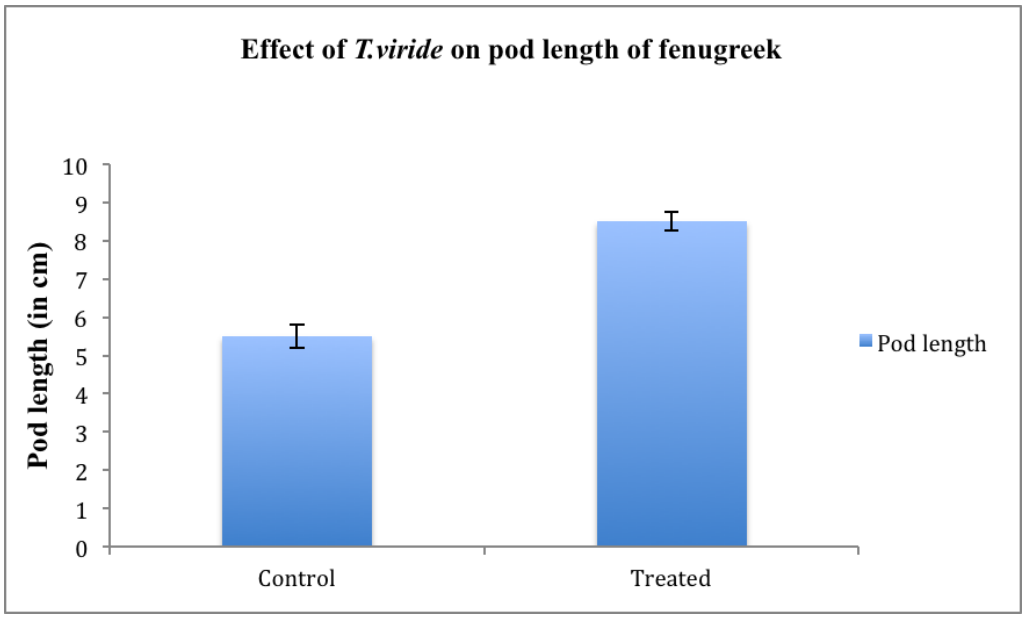



Fig 11: Effect of *T.viride* on pod length of fenugreek

Table 4: Effect of *T.viride* on yield (number of seeds per pod) of fenugreek

| Sample | Yield (number of seeds per pod) |
|---|---|
| Control | 5.75 ± 0.94 |
| Treated | 11.25 ± 0.67 |

Values represent mean (n=100) yield (number of seeds per pod) ± SE; ANOVA *p* value < 0.005

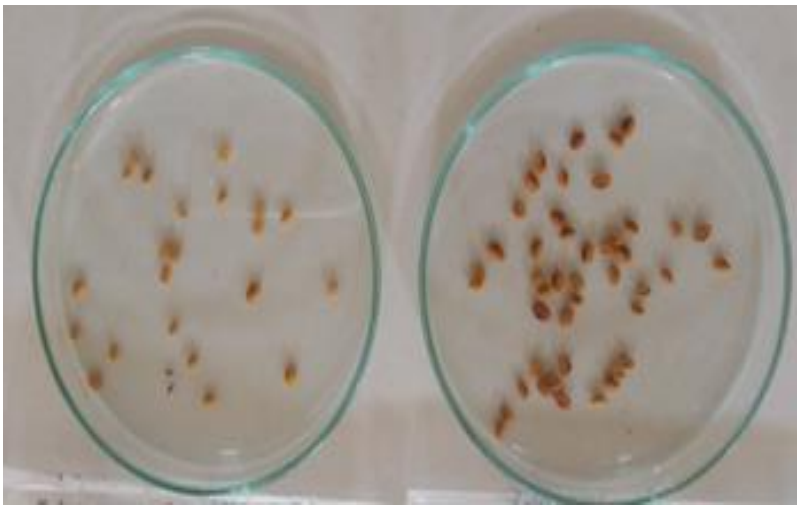



Fig 12: Fenugreek seeds of control and treated plants

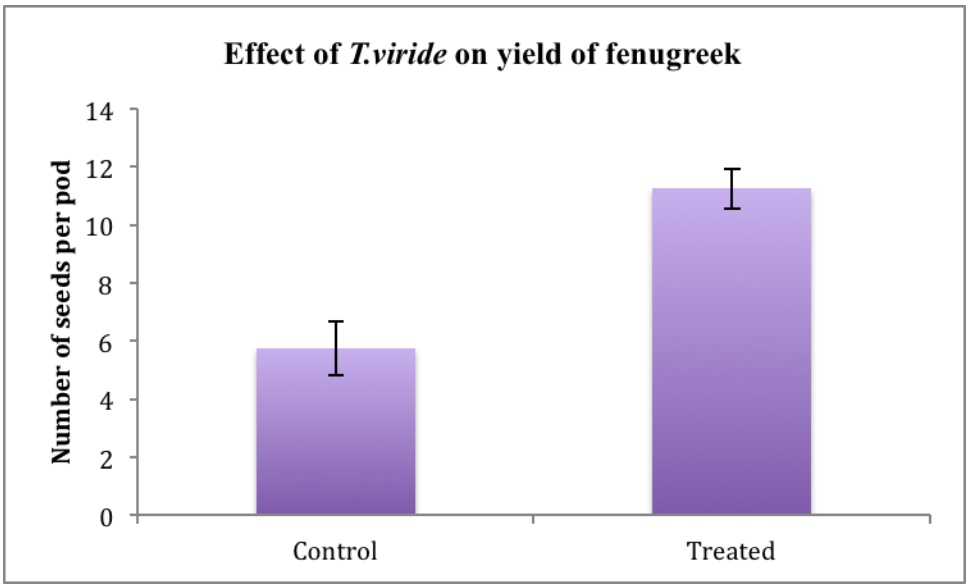



Fig 13: Effect of *T.viride* on yield (number of seeds per pod) of fenugreek

**Chlorophyll content**

The mean value of chlorophyll a, b and total chlorophyll of treated were 6.68, 2.46 and 9.14, positive control were 5.33, 2.30 and 7.63 and of control were 5.64, 1.86 and 7.5 (mg/gm fresh weight) respectively (Table 5). The amount of chlorophyll a increased by 25 %, chl b by 7 % and total chlorophyll by 19.79 % in treated plants over positive control and by 18.43 % (chl a), 32 % (chl b) and 21.86 % (total chlorophyll) over control plants (Figure 14).

Table 5: Effect of *T.viride* on chlorophyll content of fenugreek

| Sample | chl a | chl b | Total chlorophyll |
|---|---|---|---|
| Control | 5.64 ± 0.321 | 1.86 ± 0.35 | 7.5 ± 0.40 |
| Positive control | 5.33 ± 0.59 | 2.3 ± 0.34 | 7.63 ± 0.33 |
| Treated | 6.68 ± 0.22 | 2.46 ± 0.40 | 9.14 ± 0.51 |

Values represent mean (n=9) chlorophyll content ± SE; ANOVA *p* value < 0.005

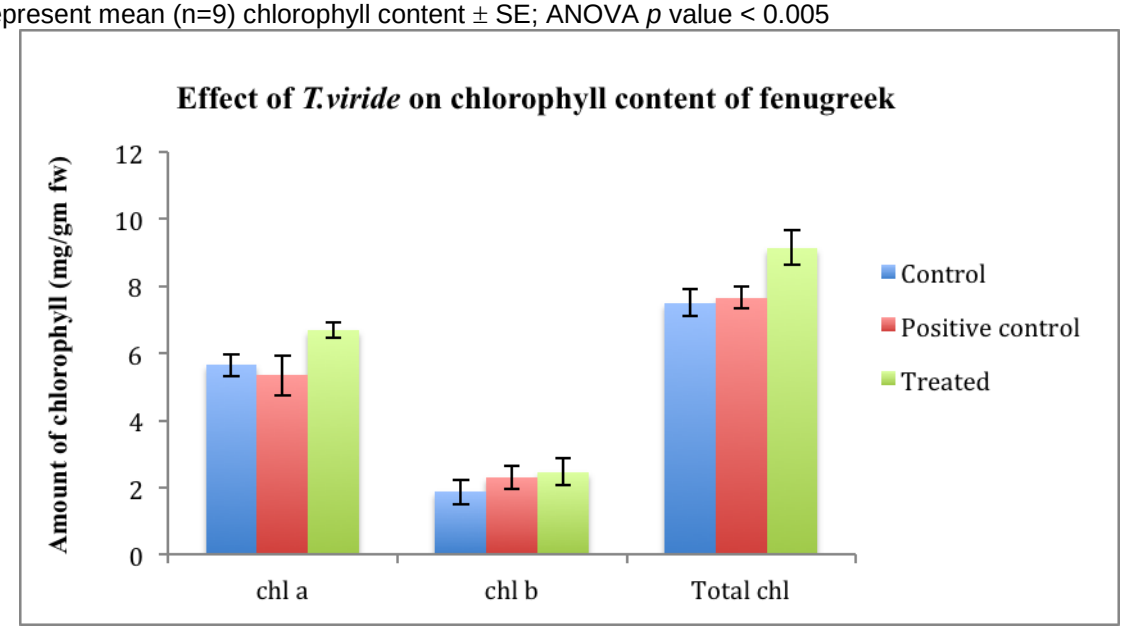



Fig 14: Effect of *T.viride* on chlorophyll content of fenugreek

**Total nitrogen in plant**

The observed titer value for control, positive control and treated samples are 2.3 ml, 4.5 ml and 5.3 ml respectively. The amount of total nitrogen in sample is calculated as discussed under 3.5. The total nitrogen content in control was 3.22 mg, positive control is 6.3 mg and treated was 7.14 mg (Table 6). The total nitrogen content in plants increased by 121.73 % in treated over control and by 13.33 % in treated over positive control (Figure 15).

Table 6: Effect of *T.viride* on total nitrogen content of plant

| Sample | Total nitrogen |
|---|---|
| Control | 3.22 ± 0.35 |
| Positive control | 6.3 ± 0.39 |
| Treated | 7.14 ± 0.59 |

Values represent mean (n=9) total nitrogen content ± SE; ANOVA *p* value < 0.005

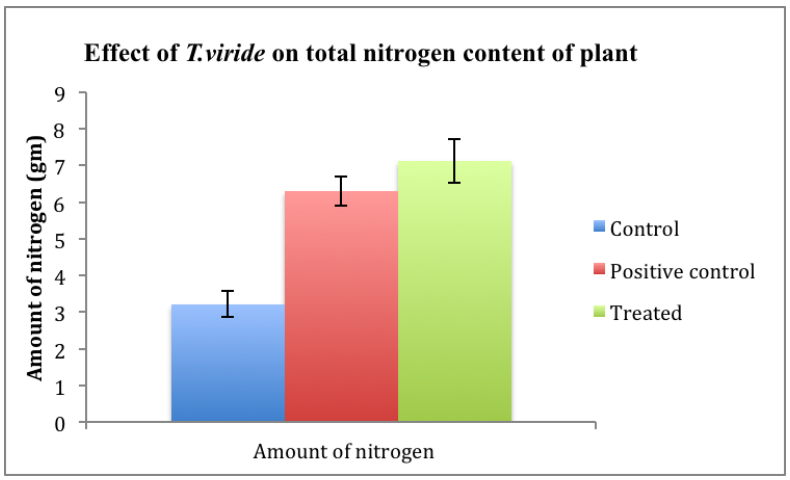



Fig 15: Effect of *T.viride* on total nitrogen content of fenugreek

**4-hydroxyisoleucine content**

The amount of free amino acids present in control and treated samples are 1.88 mg and 2.3 mg. The amount of 4-hydroxyisoleucine is calculated as discussed previously. 4- hydroxyisoleucine content in control sample was found to be 1.44 mg and 1.84 mg in treated sample (Table 7). An increase of 27.77 % of 4-hydroxyisoleucine content was observed in treated compared to control (Figure 16).

Table 7: Effect of *T.viride* on 4-OHIleu content of fenugreek

| Sample | 4-OHIleu content |
|---|---|
| Control | 1.44 ± 0.15 |
| Treated | 1.84 ± 0.17 |

Values represent mean (n=9) 4-OHIleu content ± SE; ANOVA *p* value < 0.005

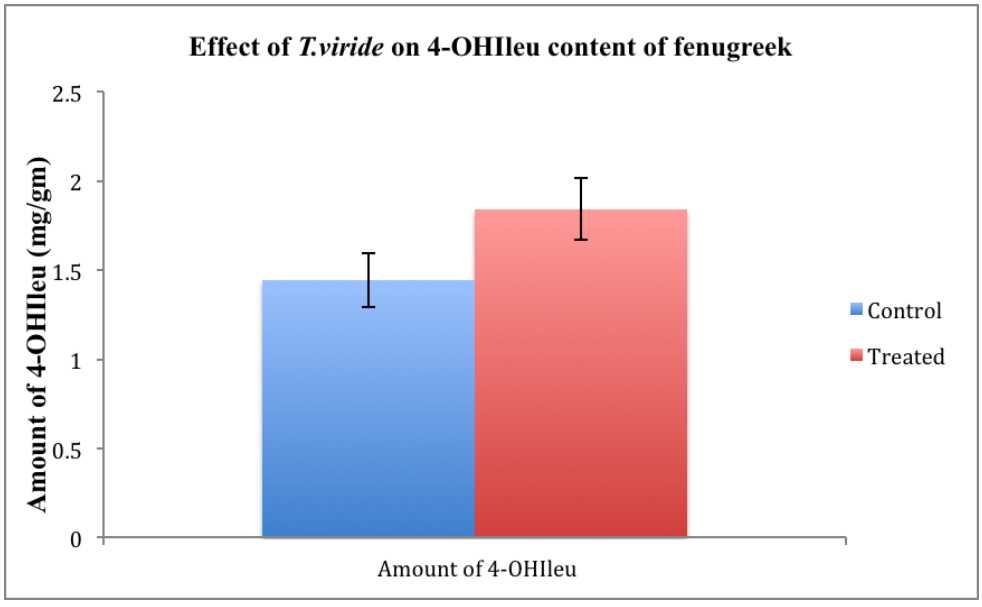



Fig 16: Effect of *T.viride* on 4-OHIleu content of fenugreek

## DISCUSSION

Previous studies in our laboratory brought to light that treatment of leguminous plants with *T.viride* TV 10 has significantly increased the rhizosphere competence of *T.viride* and has enhanced the root growth and penetration. The present study identified the ability of *T.viride* to enhance the 4-hydroxyisoleucine content in fenugreek plant. Lack of documented literature on attempts to enhance 4-hydroxyisoleucine content in fenugreek by any method prompted us to carry out this study.

Research in our laboratory indicated that *T.viride* treatment could increase the lateral root expansion of fenugreek plants thereby increasing nitrogen uptake from soil, which was reflected in the total nitrogen content of plants and specifically 4-hydroxyisoleucine.

There is huge market for pork insulin all over the world for treatment of diabetes. However in certain countries religious ethics prohibit use of pork or products derived from it. In addition use of insulin leads to a condition called insulin resistance, which further complicates the situation in a diabetes patient. On the other hand 4-hydroxyisoleucine is more effective in controlling diabetes as described in introduction. Attempts to increase nitrogen uptake in fenugreek have been reported in literature; however no study reports significant success.

### Nitrogen uptake studies documented in literature

Effect of *T.viride* on plant nitrogen uptake has been well documented in literature. It was manifested that *Trichoderma* usage can diminish the amount of nitrogen (synthetic) fertilizers by nearly 40 to 50 % without affecting the yield of the crop (Harman, 2011).

In a study conducted by Domiguez *et al*., (2016) they attributed higher nitrogen availability in *T.harzianum* treated tomato plants reflected by high amidase activity. However nitrogen uptake in legumes differs from other plants. In early stages the uptake of nitrogen is low because the plant is more dependent on the nitrogen present in the plant. The nitrogen in the seed is enough to sustain the seedling growth in the plant life cycle for a crucial period (Herdina and Silsbury, 1990). This is where the legumes differ from the non-legumes. Legumes are known for late consumption of tremendous nitrogen. Soil nitrogen uptake is directly interrelated with the lateral root extension. Branching of roots during early stages facilitate better nitrogen uptake. In a study carried out by Dayoub *et al*., (2017) they have reported that soil nitrogen availability could not alter the root depth penetration but could alter the root lateral expansion. Our results are in agreement with thesis. We observed a better lateral root expansion. The root: shoot ratio have been reported to be reduced with high availability of soil nitrogen by Corre-hellow and crozt, in 2005. However our studies did not exhibit such a reduction in root: shoot ratio.

Attempts to treat fenugreek plants to induce nodule formation by treating with rhizobium have not been successful. At high levels of soil nitrogen nodulation is observed to be low. However in faba beans and soybean nodulation was observed under nitrogen availability in soil. Dayoub *et al*., (2017) have demonstrated that nitrogen availability was not the factor influencing nodulation in legumes. Rather it was root exploration and early biomass. For the same reason fenugreek could not exhibit nodulation when treated with rhizobium.

### Molecular crosstalk between *t.viride* and root rhizosphere

*Trichoderma* sp. colonizes plant roots not only externally but also internally. The interaction between *Trichoderma* and plant is a result of chemical signal interplay from both plant and fungus. *Trichoderma* sp. is known to produce and regulate hormonal signal so as to facilitate colonization of roots. Auxins are known to be produced which directly promote root growth. In our studies, we have observed a significant difference in the root growth of treated plants compared to control plants. Not only did the lateral roots grow extensively, there was also nodule formation observed in one of the plants. Fenugreek does not form nodules like other plants. The formation of nodule clearly indicates some molecular cross talk between the plant and fungus. Such nodulation was not seen in control plants and in NPK treated plants. *Trichoderma* sp. produce expansin like proteins, which possess cellulose binding ability, and endopolygalacturonase, which help in root extension (Brotman *et al*., 2008, Moran Diez *et al*., 2009). Use of NPK only makes nitrogen available in the soil but does not affect the nitrogen transport system. The root penetration is the prerequisite for nitrogen uptake. Simply increasing the nitrogen content in soil does not necessarily increase nitrogen content in plant.

**Comparison with chemical nitrogen fertilizer treatment**

Treatment of fenugreek with chemical fertilizers exhibited higher nitrogen and amino acid content in plants (Li *et al*., 2017). However it was not higher than that in plants administered with *T.viride.* There was a 13 % increase in total nitrogen in plants nursed with *T.viride* compared to NPK treated plants and 121 % increase in total nitrogen over control plants respectively.

High nitrogen levels supplement in the soil have been reported to increase the pest attack (Han *et al*., 2013). Singh *et al*., in 2005 illustrated similar works on sorghum seedlings. This probably could be attributed to the fact that high nitrogen in plant is accumulated in the form of free amino acids (Sogawa, 1982). The proteins needed by insect are often different from the protein present in the plant (Schoonhoven, 2005). Hence availability of free amino acids makes protein synthesis in insect less energy consuming. On the other hand nitrogen in the form of protein requires to be first broken into individual amino acids before being incorporated into protein in a desired sequence.

Most organic fertilizers, bio fertilizers and *T.viride* facilitate relatively slow and continuous uptake of nitrogen from soil (Naguib, 2011) thereby maintaining nitrogen in the plant protein form. Therefore rise of pest attack is significantly reduced in plants treated with *T.viride.*

## CONCLUSION

This study was aimed to observe the effect of *T.viride* on growth parameters *viz* root and shoot lengths, chlorophyll and total plant nitrogen and yield of fenugreek plants.

This study has confirmed the effect of *T.viride* TV 10 on growth parameters viz root and shoot lengths, chlorophyll and total plant nitrogen and yield of fenugreek plants. A 25 % increase in root lengths and 2-fold increase in lateral root expansion were observed in *T.viride* administered plants over control. An outstanding 50 % growth in shoot length was observed over control. The total chlorophyll in treated plants increased by 19.79 % over positive control and 21.86 % over control plants. The treated plants exhibited 9-fold increase over control in total plant nitrogen content and by 13.3 % over positive control plants.

*T.viride* treatment has proved to be potent in enhancing the 4-hydroxyisoleucine content in fenugreek. A 27 % enhancement of 4-hydroxyisoleucine content in treated fenugreek over control plants. Fenugreek seed inoculation for 24 hours in *T.viride* suspension enhanced the growth and yield parameters and 4-hydroxyisoleucine concentrations in fenugreek.

## REFERENCES

Alaoui, S.B., 2005. *Trigonella foenum-graecum*. Ecoport database.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in beta vulgaris. *Plant Physiology*. 24, 1-15.

Baboo, R.,1997. Effect of cutting management, nitrogen and phosphorous on growth and yield of fenugreek (*Trigonella foenum - graecum* L.). *Ann. Agri. Res*.18 (3), 380 - 382.

Baskin and Baskin, C.C., Baskin, J.M., 2001. Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination. Academic Press, San Diego, CA, USA. 666 s.

Basu, S.K., 2006. Seed Production Technology for Fenugreek (*Trigonella foenum-graecum* L.) in the Canadian Prairies (thesis). University of Lethbridge, Faculty of Arts Sci., Lethbridge, Alberta, Canada.

Bellostas, N., Hauggaard-Nielsen, H., Andersen, M.K., Jensen, E.S., 2003. Early Interference dynamics in Intercrops of Pea, Barley and Oilseed Rape. *Biol. Agric. Hortic.* 21, 337–348.

Benitez, T., Rincon, A.M., Limon, M.C., Codon, A.C., 2004. Biological control mechanisms of *Trichoderma* strains. *Int. J. Microbiol*. 45, 249- 260.

Betty, R.I., 2008. The many healing virtues of fenugreek. Spice India. 1, 17-19.

Broca, C., Manteghetti, M., Gross, R., Baissac, Y., Jacob, M., Petit, P., Sauvaire, Y., Ribes, G., 2000. 4-Hydroxyisoleucine: Effects of synthetic and natural analogues on insulin secretion. *Eur. J. Pharmacol. 390*, 339–345

Celar, Valic, N., 2005. Effects of *Trichoderma* spp and *Glicladium roseum* culture filtrates on seed germination of vegetables and maize. *Journal of Plant Diseases & Protection*. 112(4), 343-350.

Chaverri, P., Ghazis, R., Samuels, G.J., 2011. *Trichoderma amazonicum*, a new endophytic species on *Hevea brasiliensis* and *H.guianensis* from the Amazon basin.*Mycologia*. 103, 139-151.

Chaverri, P., Samuels, G.J., 2013. Evolution of habitat preference and nutrition mode in a cosmopolitan fungal genus with evidence of inter kingdom host jumps and major shifts in ecology. *Evolution*. 67, 2823-2837.

Che"rif, M., Benhamou.N., 1990. Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma sp*. on *Fusarium oxysporum f. sp. radicislycopersici*. *Phytopathology* 80, 1406-1412.

Corre-Hellou, G., Brisson, N., Launay, M., Fustec, J., Crozat, Y., 2007. Effect of root depth penetration on soil nitrogen competitive interactions and dry matter production in pea–barley intercrops given different soil nitrogen supplies. *Field Crops Res.*103, 76–85.

Corre-Hellou, G., Crozat, Y., 2005. Assessment of Root System Dynamics of Species Grown in Mixtures under Field Conditions using Herbicide Injection and $^{15}N$ Natural Abundance Methods: A Case Study with Pea, Barley and Mustard. *Plant Soil*. 276, 177-192.

Dayoub, E., Naudin, C., Piva, G., Shirtliffe, S.J., Fustec, J., Corre-Hellou, G., 2017. Traits affecting early season nitrogen uptake in nine legume species. *Heliyon*. 3(2), e00244.
Ecocrop, 2017. Ecocrop database. FAO, Rome, Italy.
Flammang, A., Cifone, M., Erexson, G., Stankowski, L., 2004. Genotoxicity testing of a fenugreek extract. *Food Chem. Toxicol*. 11, 1769–1775.
Fowden, L., Pratt, H.M.S., Mith, A., 1973. 4-Hydroxyisoleucine from seed of *Trigonella foenum-graecum*. *Phytochemistry. 12*, 1707–1711.
Fuller, S., Stephens, J.M., 2015. Diosgenin, 4-Hydroxyisoleucine, and Fiber from Fenugreek: Mechanism of Actions and potential Effects on Metabolic Syndrome. *Adv. Nutr. 6*, 189–197.
Geurts, R., Bisseling, T., 2002. *Rhizobium* Nod Factor Perception and Signalling. *The Plant Cell*. 14, s239-s249.
Han, P., Lavoir, A.V., Le Bot J., Amiens-Desneux E., Desneux, N., 2014. Nitrogen and water availability to tomato plants triggers bottom-up effects on the leaf miner *Tuta absoluta*. *Sci. Rep*. 4, 4455.
Harman, G.E., 2000. Myths and dogmas of bio control-changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant disease*. 84, 77-393.
Harman, G.E., 2011. Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. *New Phytologist*. 189, 647–649.
Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. *Trichoderma species* - opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*. 2, 43–56.
Herdina, J. H., Silsbury, J. H., 1990. Nodulation and Nitrogen Fixation of Faba Bean (*Vicia faba* L.) as Affected by Removal of the Cotyledons and Nitrate Supply. *Annals of Botany*. . 69 (3), 227-230.
Herdina, Silsbury, J.H., 1990. Growth, nitrogen accumulation and partitioning, and $N_2$ fixation in faba bean (*Vicia faba* cv. Fiord) and pea (*Pisum sativum* cv. Early Dun) Field Crops Res. 24,173–188.
Hjeljord, L. G., Stensvand, A., Tronsmo, A., 2000. Effect of temperature and nutrient stress on the capacity of commercial *Trichoderma* products to control *Botrytis cinerea* and *Mucor piriformis* in greenhouse strawberries. *Biolog Control*, 19, 149-160.
Howell, C.R., Hanson, E.L., Stipanovic, R.D., Puckhaber, L.S., 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens. Phytopathology* 90, 248–252.
Kjeldahl, J., 1883. Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern (New method for the determination of nitrogen in organic substances). *Zeitschrift fur analytische Chemie*. 22(1), 366-383.
Lakshmi, J., Gowda, R., Parashivamurthy, Narayanaswamy, S., Shivanandan, V.N., 2015. Influence of pre–flowering pinching and maleic hydrazide spray on plant growth, seed yield and quality attributes in fenugreek. *Legume Research*. 38(3), 353 - 357.
Li, X., He, P., Xu, J., Fu, G., Chen, Y., 2017. Effect of nitrogen and phosphorus on growth and amino-acid contents of *Porphyra yezoensis*. *Aqua. Res*. 48, 2798– 2802.
McCormick, K., Norton, R., Eagles, H.A., 2006. Fenugreek has a role in south-eastern Australian farming systems. In: Proceedings of "Groundbreaking stuff", 13th Annual Agronomy Conference, Perth, Australia, 639.
Mehrafarin, A., Qaderi, A., Rezazadeh, S.H., Naghdi-Badi, H., Noormohammadi, G.H., Zand, E., 2010. Bioengineering of important secondary metabolites and metabolic pathways in fenugreek (*Trigonella foenum-graecum* L.). *J. Med Plant. 9*, 1-18.
Monte, E., 2001. Understanding *Trichoderma*: Between Agricultural Biotechnology and microbial Ecology. *Int Microbiol*. 4, 1-4.
Moore, S., Stein, W.H., 1948. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem*. 176, 367-388.
Moosavi, S.G., 2014. Fennel morphological traits and yield as affected by sowing date and plant density. *Adv. Agric. Biol.* 1 (1), 45 - 49.
Moradi kor, N., Moradi, K., 2013. Physiological and pharmaceutical effects of fenugreek (*Trigonella foenum-graecum* L.) as a multipurpose and valuable medicinal plant. *Global J. Med. Plant Res.* 1,199–206.
Naguib, N.Y.M., 2011. Organic vs chemical fertilization of medicinal plants: a concise review of researches. *Adv. Environ. Biol*. 5(2), 394-400.
Naidu, M.M., Shyamala, B.N., Naik, J.P., Sulochanamma, G., Srinivas, P., 2011. Chemical composition and antioxidant activity of the husk and endosperm of fenugreek seeds. LWT-Food Science and Technology. 44, 451-456.
Nandre, D.R., Ghadge, R.G., Rajput, B.S., 2011. Effect of sowing dates and nutrient management on growth and seed yield of fenugreek. *Adv. Res. J. Crop Improv*. 2(2), 215 -220.
Pandey, H., Awasthi, P., 2015. Effect of processing techniques on nutritional composition and antioxidant activity of fenugreek (*Trigonella foenum-graecum*) seed flour. *J Food Sci Tech*. 52(2), 1054-1060.
Pelczar, M.J., Chan, E.C.S., Noel R., Kreig 1996. *Microbiology*. 553-559.
Poi, S.C., Basu, T.K., Behari. K., Srivastav, A., 1991. Symbiotic effectiveness of different strains of *Rhizobium meliloti* in selecting inoculants for improvement of productivity of *Trigonella foenum-graecum. Environ. Ecol*. 9: 286-287.
Rifai, M. A., 1969. A revision of the genus *Trichoderma. Mycological Papers.* 116: 1- 56.
Roberts, K. T., 2011. The potential of fenugreek (*Trigonella foenum-graecum*) as a functional food and nutraceutical and its effects on glycemia and lipidemia. *J. Med. Food.* 14 (12), 1485-1489.
Samolski, I., Rincon, A.M., Pinzon, L.M., Viterbo, A., Monte, E., 2012. The qid74 gene from *Trichoderma harzianum* has a role in root architecture and plant biofertilization. *Microbiology*.158, 129-138.
Sara Domínguez, M., Belén Rubio, Rosa E., Cardoza, Santiago Gutiérrez, Carlos Nicolás, Wagner Bettiol, Rosa Hermosa, Enrique Monte, 2016. Nitrogen Metabolism and Growth Enhancement in Tomato Plants Challenged with *Trichoderma harzianum* Expressing the *Aspergillus nidulans*Acetamidase amdS Gene.*Front Microbiol*. 7, 1182.
Sauvaire, Y., Ribes, G., Baccou, J.C., 1991. Implication of steroid saponins and sapogenins in the hypocholesterolemic effect of fenugreek. *Lipids*. 26, 191-197.
Schoonhoven, L. M., van Loon, J. J. A., Dick, M., 2005. Insect-plant biology. Oxford University Press, Oxford.
Shaban, M., 2013. Effect of water and temperature on seed germination and emergence as a seed hydrothermal model. *Int. J. Advanced Biol. And Biom. Res.* 1(12), 1686-1691.
Sharma, R.D., Raghuram, T.C., Rao, N.S., 1990. Effect of fenugreek seeds on blood glucose and serum lipids in type I diabetes. *Eur. J. Clin. Nutr*. 44, 301-306.
Shoresh, M., Harman, G.E., Mastouri, F., 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Re.v Phytopathol.* 48, 21-43.
Singh, B. U., Padmaja, P. G., Seetharama, N., 2005. Stability of biochemical constituents and their relationships with resistance to shoot fly, *Atherigona soccata* (Rondani) in seedling *Sorghum*. *Euphytica*. 136, 279-289.

Sogawa, K., 1982. The rice brown planthopper: Feeding physiology and host plant interactions. *Ann. Rev. Entomol*. 27, 49–73.
Sorensen, J., Sessitsch, A., 2007. Plant-associated bacteria lifestyle and molecular interactions. In J.D. van Elsas, J.K. Jansson, and J.T. Trevors (Eds.), Boca Raton, FL: CRC Press, Taylor and Francis Group. *Modern Soil Microbiology*, 2nd ed. 100, 211–236.
Srinivasan, K., 2006. Fenugreek (*Trigonella foenum-graecum*): A review of health beneficial physiological effects. *Food Rev. Int*. 22 (2), 203-224.
Srivastava, D., Rajiv, J., Mahadevamma, Naidu, M.M., Puranaik, J., Srinivas, P., 2012. Effect of fenugreek seed husk on the rheology and quality characteristics of muffins. *Food Nutr. Sci*. 3, 1473-1479.
Thorup-Kristensen, K., 2001. Are differences in root growth of nitrogen catch crops important for their ability to reduce soil nitrate-N content, and how can this be measured? *Plant Soil*. 230, 185–195.
Tuncturk, R., Celen, A.E., Tuncturk, M., 2011. The effect of nitrogen and sulphur fertilizers on the yield and quality of fenugreek (*Trigonella foenum - graecum* L.), *Turkish J. of Field Crops.* 16(1), 69 – 75.
Yedida, I., Benhamou, N., Chet, I., 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.* 65, 1061-1070.
Yoshikawa, T., Toyokuni, S., Yamamoto, Y., Naito, Y., 2000. Free radicals in chemistry biology and medicine. OICA International, London.