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Examining the Effects of Nitrogen Fixing Plants on the Growth of *Solanum Lycopersicum* and *Cucurbita Maxima*

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Abstract

The increases in population forces farmers and researchers to derived new ways to enhanced crop production, one of them is the use of chemical fertilizers although their hazardous effects on our ecosystem are enormous, in current research a simple and alternative method is adopted to enhanced crop yield i.e. intercropping with legumes in which, effects on the growth of *Cucurbita maxima* and *Solanum lycopersicum* while intercropping with *Vigna unguiculata subsp. unguiculata* (ability to fixed nitrogen) was studied, and it has been estimated that *Vigna unguiculata subsp. unguiculata* shows positive impact on *Cucurbita maxima*, growth i.e. it enhances its shoot length, leaf area, root length, average growth rate and chlorophyll content but the results are negligible with *Solanum lycopersicum* in which the growth rate is same as in control and in some cases the growth rate decreased therefore, it is concluded that even if a plant is nitrogen fixing it gives different results with different other non-legumes and if we found out which legume specie gives better productivity when intercropped with other important cereals we can limit the usage of chemical fertilizers and also meet the food demand of over populated world without any hazardous effects of chemicals.

Introduction

Nitrogen is regarded as the most essential nutrients for plants growth as major components of plants i.e. amino acids, ATP, chlorophyll, nucleic acids all have nitrogen as a major part of their structures. Nitrogen when supplied optimally has the ability to increases plants leaf area production and duration, photosynthetic process and net assimilation rate [1]. Deficiency of nitrogen lead to reduced plant growth, chlorosis i.e. yellowing of plants leaves, lateral bud growth also get affected, purple or red spots may seems on leaves surface with nitrogen deficiency, hence all plants i.e. cereals, fibers, horticulture plants all required a balance quantity of nitrogen for rapid and improved growth and development, in Pakistan nitrogen is majorly used for countries cash crops such as wheat, rice, sugarcane and cotton.

To fulfill the nitrogen requirement of plants farmers started using nitrogen fertilizers since long ago as they had the potential to fulfill the food requirements of a huge population by working fast and readily available to plants in large quantities, but on contrary to this chemical fertilizers lead to the deterioration of our atmosphere to a greater extent. One of the most important issues regarding the use of chemical fertilizers is its

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ground water contamination i.e. nitrogen is so water soluble that when it leach out of the soil it drains into ground water and because of that not only would the plants not really be getting the nutrient they need, the nitrogen in ground water led to many health problems and birth defects in animals and humans using the water. At the University of Wisconsin, Madison, they discovered altered neurological, endocrine and immune system in fetus and in mice due to the effects of chemical fertilizers. These alteration impact the ability to learn and aggregation patterns [2]. Also because of the overload of these chemical fertilizers, soil microbes may produces high of nitrous oxide which act as a greenhouse gas in our environment and has an ability to trap heat 300 times more than carbon dioxide. (Mole, 2014)[3]. Other than that they can cause soil detoriation by soil acidification, nitrogen has a major role in soil acidification [4].

Because of many adverse effects of chemical fertilizers that can't be ignored organic fertilizers are used as an alternatives of chemical fertilizers because they are involved in enhancing soil fertility, in improving soil water retention property i.e. compost contains an astonishing variety of microbes which may be beneficial in controlling pathogens and also in converting complex compounds into simpler one hence enhance soil fertility. But nitrogen in organic fertilizers in some way leached out or get volatized due to which the final nitrogen content left low, so this need some modifications i.e. farmers can't use them directly in their fields but after these modifications nutrients content in organic fertilizers left low [5].

One form of organic fertilizers is bio fertilizers i.e. use of living organisms as a fertilizer, which proves to be an astonishing way to fulfil nutrient requirement, include living bacterial, algal and fungal inoculant alone or in combination, which improves the nutrient availability of plants. Some of these bacteria's are free living while some forms symbiotic relationship with leguminous plants. Species of *Azospirillum, Azotobacter, Beijerinckia* (i.e, microorganisms that establish associations with grass plants), *Rhizobium*, *Bradyrhizobium,* and *Azorhizobium* (i.e, bacteria establishing symbiosis with legumes), Frankia (i.e, symbiotic actinomycetes with woody plants), Nostoc (i.e, blue-green algae establishing symbiosis with different plants) or *Anabahena* (i.e, ferns) are found to forms symbiotic relations with the purpose to fix nitrogen [6]. These diazotrophs usually the rhizobia forms root nodule with many legume plants specie hence affecting its growth. It has been seen that these rhizobia proves to be increasing legumes crop yield while given alone or in combination with other strains [6]. Legume rhizobia symbiosis is responsible for at least 70 million tons of N per year [7].

To use rhizobia for non-leguminous plants we have to go through many biotechnological modifications i.e. many researchers tried to associate non-leguminous plants with nitrogen fixing bacteria's, many other tried to produce genetically modified plants having ability to produce nitrogenase, other tried to improve nitrogen fixing bacterial strains but did not get successful because there are many constraint in the way such as improved bacterial strain does not give desirable outcomes when inoculating in the field with other microflora, other than slow growth of some diazotrophs is also a limiting factor because they then difficult to culture, some others proves pathogenic toward human so we limit their use in lab [8], when transfer genes from paenibacillus in E.coli only 10% nitrogenase activity was observed to enhance this activity total 28 genes were selected and in result nitrogenase activity reached 50.1% [9], which was also not satisfactory as compared to natural nitrogenase activity. So to make nitrogen fixing bacteria usable for non-leguminous plants, required extensive experimentation and labour.

An alternative way to promote the growth of non-legume crops is by growing them with legumes having symbiosis relation established a process termed as intercropping. Legume in rotation serves as an improved way to enhanced crop productivity and also to improve soil fertility, in maize - legume rotation is was analyzed that velvet bean enhanced the productivity of maize as compared to soybean and cowpea [10], likewise by intercropping cowpea, soybean, and ground nut with maize it revealed that cow pea and ground nut performed better than soybean in enhancing crop yield and improving soil fertility [11], rice also shown high productivity when rotate with winged bean and long bean with minimum or no usage of nitrogen fertilizers [12], other than that legumes in cropping system also has positive influences on environment i.e. if we switch towards legume usage we can minimized nitrous oxide emission in environment compared to chemical fertilizers [13], so legume shows positive effects not only in enhancing other crops productivity but also act as environmentally friendly as compared to chemical fertilizers, to make our agricultural system sustainable we have to shift towards using legumes in our agricultural systems.

In this work *Vigna unguiculata subsp*. *unguiculata* is used as a nitrogen fixing plant, intercropped with *Cucurbita maxima*, and *Solanum lycopersicum* to estimate the differences in their growth with respect to control.

Methods and material

Experimental design

The experiment was conducted on two non-legumes plant species i.e. *Cucurbita maxima* and *Solanum lycopersicum*, each having two control groups and one experimental group. One control group includes plant species grown alone in pots, and second control group includes plants specie grown with *Laburnum alpinum* while the experimental group includes growing plants species with *Vigna unguiculata subsp*. *unguiculata*, (a legume). All these groups are set to grow with three replicates, in pots with a diameter of 12 inches, length of 8 inches and having 500 grams soil. All plants seeds are taken from same source, and sown one inches deep in soil, in a way that each replicate contains two seeds of test plants species. *Laburnum alpinum* and *Vigna unguiculata subsp*. *unguiculata* were first grown for about two months then used as a treatment for *Cucurbita maxima* and *Solanum lycopersicum*. At the end of experiment different parameters were considered and compared i.e. plants shoot length, leaf area, average growth rate, root length and chlorophyll content.

Shoot length measurement

The shoot length of plantlets were measured by using scale, during plant growth and at the end of experiment. During plant growth when in the pot, shoot length were measured from plant base to its tip and at the end of the experiment plants from the replicates of each group were taken out carefully to avoid root disruption, from the soil and then shoot measurement were done by again setting the ruler at the base of the plant to its tip.

Leaf area calculations

For the calculation of leaf area, the best grown plants from each replicate was selected and then average of product of leaves height and width was recorded. Total numbers all leaves were also noted (Figure 1 and 2).

Figure 1: Measurement of leaf length.

Figure 2: Measurement of leaf width.

Root length

The root lengths were calculated by image J software [14]. For that purpose, the best-grown plants from each replicates were chosen and gently removed from soil. Then the roots were dipped in water and move gently to remove soil particles from it. After that, they were placed on a white background paper and a picture was clicked to analyze in image J software. To use the image J software first set the scale by 1 cm distance in pixels and then measure the root lengths. After measurements, a mean root length value for each group was calculated (Figure 3A).

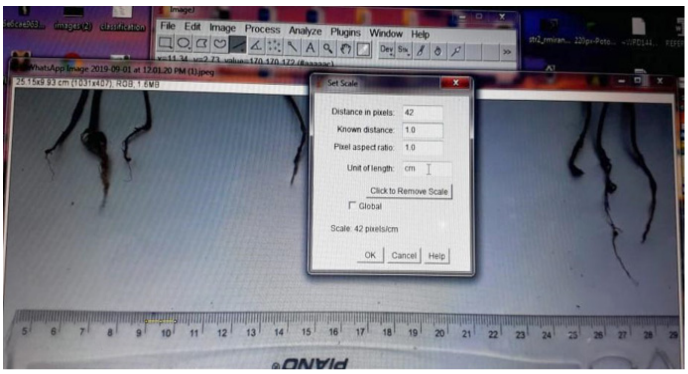

Figure 3A: Measuring root length of *S. lycopersicum* by image J.

This image shows the use of image J for root length calculations. The first step in making calculations is to set the scale the yellow line on scale between 9 to 10 cm shows the use of straight line tool in setting the scale according to pixels that should be 1cm and converting the unit into cm. Now the image J is ready to measure different root lengths according to the pixels accurately

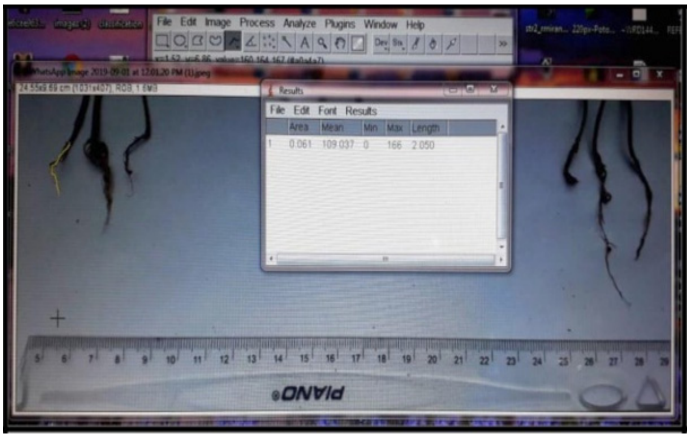

Figure 3B: Measuring root length of *S. lycopersicum* by image J.

These figures shows that after setting the scale we can easily calculate the root lengths by simply using free hand tool on image J. The yellow line indicates the length of plant root grown with V.unguiculata subsp. unguiculata replicate 1 which is 2.050cm, hence in the same way we calculate the root lengths of all group replicate (Figure 3B).

Average growth rate

The average growth rate was calculated by the formula =

$$\frac{(S_2 - S_1)}{T}$$

(Where S_1 is the first reading of plant height, S_2 is the 2nd reading and t is the time between first and 2nd reading.)

Growth curve

Growth curve represent the growth of plants on the basis of every day observation, for making a growth curve observation on a regular basis were noticed and a graph was plotted.

Chlorophyll content

Chlorophyll content of plants tells about a plants physiological conditions, plants nutrient status etc. that's why many researchers focuses on devising new and better methods and formulas for the calculation of chlorophyll content of plant, some of formulae for calculating chl a, chl b, was devised by Arnon in 1949 (commonly used).

In this procedure we first cut leaves area of about 1cm^2 and then cut that 1cm^2 leave part into smaller pieces. After that mashed it with 80% acetone in pestle and mortar until leaves pieces give off its color in acetone and mashed completely then we add some more acetone, so that the total volume reached 25ml then centrifuge it at 2000rpm for 5min. Calculate its absorbance by using spectrophotometer at 663nm and 645 nm and calculate chlorophyll content by using Arnon equations (Figure 4,5 and 6):

Chlorophyll a (µg/ml) = 12.7 (A663) - 2.69 (A645)

Chlorophyll b (µg/ml) = 22.9 (A645) - 4.68 (A663)

Total chlorophyll (µg/ml) = 20.2 (A645) + 8.02 (A663)

Figure 4: Area of plants for chlorophyll content determination.

'A' shows $1cm^2$ leaf part of S.lycopersicum while B shows $1cm^2$ leaf part of *C.maxima*.

Figure 5: Mashing leaf in 80% acetone.

In this figure 'A' shows the conversion of leaf ($1cm^2$ part) into smaller pieces and 'B' shows its mashing in little amount of acetone.

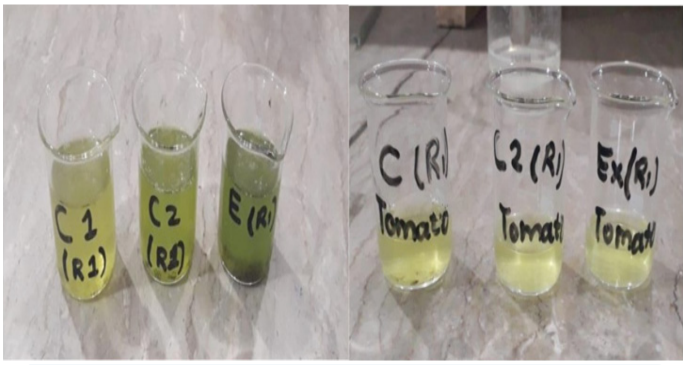

Figure 6: A suspension of different groups, leaf parts in acetone.

Figure 'A' shows the suspension of C.maxima after mashing its leaf pieces in acetone where C1 shows the suspension from control group (replicate 1), C2 shows suspension from plant grown with L.alpinum (replicate1) and E shows the suspension from plants grown with V.unguiculata subsp. unguiculata (replicate1).

While figure 'B' shows the suspension from S.lycopersicum groups. After calculating the chlorophyll content of replicate 1 plants we repeat the procedure with rep.2 and rep.3 and then calculate the mean chlorophyll content of all groups.

Results & discussions

Shoot length

By calculating the shoot length of different groups, following results were obtained (Table 1):

Table 1: Comparison between the shoot lengths of different *C. maxima* groups.

Mean shoot length of *Cucurbita maxima* (control)	Mean shoot length of *Cucurbita maxima* grown With *Laburnum alpinum* (control)	Mean shoot length of *Cucurbita maxima* grown With *Vigna unguiculata sub sp. unguiculata*
4.6 inches	5.1 inches	6.06 inches

Shoot length enhancement always correlate with effective growth of plant, and it depends on nutrients availability, on soil condition, soil pH, suitable environmental conditions for a specific plant species, moisture, on the use of nitrogen and plant nitrogen uptake ability. The above comparison shows shoot length enhancement of *C.maxima* when grown with *V.unguicalata subsp*. *unguiculata*, the reason behind this is the availability of nitrogen to *C.maxima* provided by V.*unguiculata subsp*. *unguiculata* symbiotic relation with rhizobia, higher nitrogen content (i.e. of 250 kg ha^{-1}) presumably has a positive effects on the growth of *Cucurbita maxima* shoot length. A four year experiment on pumpkin with different nitrogen concentrations also showed enhanced shoot length [15] (Table 2).

Table 2: Comparison between the shoot lengths of different *S. lycopersicum* groups.

Mean shoot length *of Solanum lycopersicum* (control)	Mean shoot length of S. *Lycopersicum* with *Laburnum Alpinum* (control)	Mean shoot length of *S. lycopersicum* with *V. unguiculata subsp. unguiculata*
4.0 inches	3.8 inches	3.4 inches

From the above comparison it seems that S.lycopersicum did not go well when planted with nitrogen fixing V.*unguiculata* subsp. *unguiculata* the reasons behind this are negative regulation of nitrogen uptake (i.e. inhibition in nitrogen uptake during early plants stages), S.lycopersicum behavior towards a legume and may be because of the competitive environment due to the presence of more plants in the same pot. This compensation sort of behavior in which yield of a plant is lowered than expected, was seen by many other researchers and they also conclude that S.lycopersicum intercropping with common beans is not significant [16]

Leaf area calculations

The results of leaf areas calculations of *C.maxima* are as follows:

Table 3: Comparison between the leaf areas of different groups.

Groups	Average number of leaves	Average leaf area
Control	3.67	0.561 inches
Grown with *L.alpinum*	2.3	0.624 inches
Grown with *V.unguiculata subsp. unguiculata*	3.67	0.7548 inches

Leaf area calculation for S.lycopersicum was not possible because of the small leaf sizes.

The growth of plants leaves is depends on amino acid activities. Amino acids shows different activities in different plants leaves and all those amino acids required nitrogen for their synthesis. As nitrogen is the most essential part of their structures, hence we can assume that increased nitrogen availably led to increase leaf area because of increase amino acids synthesis (Table 3). The above table display an increase leaf area of *C.maxima* when intercropped with V.*unguiculata* subsp. *unguiculata* and the reason behind that is the increase supply of nitrogen because of the symbiotic relation of V. *unguiculata* subsp. *unguiculata* which ultimately led to the synthesis of more amino acids hence increases plant leaf area.

Root length calculations

Table 4: Comparison between the root lengths of *C. maxima* groups.

Groups	Mean root lengths
Control	1.689 cm
Grown with *L.alpinum*	2.271 cm
Grown with V.unguiculata subsp. unguiculata	3.299 cm

Table 5: Comparison between the root lengths of *S. lycopersicum* groups.

Groups	Mean root lengths
Control	3.43 cm
Grown with *L.alpinum*	3.389 cm
Grown with *V.unguiculata subsp. unguiculata*	2.473 cm

Root length comparison also displayed the same results as in shoot length comparison i.e. in *C.maxima* root length increases when intercropped with both legumes *L.alpinum* and V.*unguiculata* subsp. *unguiculata*, while the results were insignificant in the case of S.lycopersicum the reasons behind this is basically a positive nitrogen uptake at early plant stages by *C.maxima* while a negative nitrogen uptake and a competitive behavior by S.lycopersicum when intercropped with legumes(Table 4 and 5) (Figure 7,8 and 9).

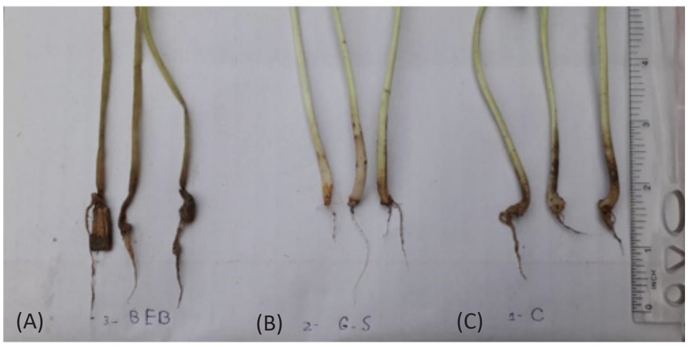

Figure 7: A root length comparison between the groups of *C. maxima.*

This figure shows the root length of different *C. maxima* groups. 'A' shows the replicates of plants grown with V.unguiculata subsp. unguiculata. 'B' shows the replicates grown with *L.alpinum* while 'C' shows the control group where the plants grown alone.

Figure 8: A root length comparison between the groups of *S. lycopersicum*

This figure shows the root length of different S. lycopersicum groups. 'A' shows the replicates of plants grown with V.unguiculata subsp. unguiculata. 'B' shows the replicates grown with L.alpinum while 'C' shows the control group where the plants grown alone.

Figure 9: Root length measurement of *C. maxima*.

This figure shows the working of image J software in which root lengths calculation of C.maxima plants can be done by selecting a free line tool which is represented in yellow line in this figure.

Average growth rate

Table 6: Comparison between the average growth rates of *C. maxima.*

Groups	Average growth rate
Control	0.28
Grown with *L.alpinum*	0.306
Grownwith V.unguiculata subsp. unguiculata	0.36

Table 7: Comparison between the average growth rates of *S. lycopersicum.*

Groups	Average growth rate
Control	0.23
Grown with *L.alpinum*	0.22
Grownwith*V.unguiculata subsp. unguiculata*	0.2

Plant growth rate defines the overall health of a plant and it highly affected by nutrients uptake, temperature, light and water. In this case all groups are provided with the same amount of water, temperature and light but the nutrient (nitrogen) availability is different in control and experimental group because of that in above Table 6 growth rate increases when *C.maxima* was intercropped with legumes while in Table 7 no growth rate enhancement was seen in S.lycopersicum groups when intercropped with legumes, because of S.lycopersicum competitive and negative behavior with V.*unguiculata* subsp. *unguiculata*.

Growth analysis curve

Growth analysis curves represent the overall growth of plants; every day observation was used in processing growth analysis curve.

Table 8: Mean growth observations of *C. maxima* groups.

Days	Control (solely)	Control with L.alpinum	Treatment With V.unguiculata subsp. unguiculata
11	Nothing observed	Nothing observed	Nothing observed
12	Nothing observed	Nothing observed	Nothing observed
13	2 seeds sprout	1 seed sprout	3 seed sprout
14	Got bigger about 0.5-0.7inches long	0.5 inches long	0.6-0.9 inches long
15	0.5-0.9 inches long	0.8 inches long	0.6-1.0 inches long
16	1 new seed sprout Overall length = 0.4-1.2	Plantlet height increase to 1.7 inches	0.9-1.5 inches long
17	0.7-1.5 inches	1 new seed sprout Overall length= 0.5-2.3	2 seed sprout 0.6-2 inches long
18	2 new seed sprout Overall length = 0.5-2 inches	3 new seed sprout Overall length= 0.6-4 inches	1 new seed sprout Overall length =1-2.5 inches
19	1 new seed sprout 0.3-2.9 inches	1.4-4.4 inches	1.5-3.1 inches long
20	0.5-4 inches	2.5-4.9 inches	2.5-4.5 inches long
21	1.0-4.5 inches	4.0-5.0 inches	3.2-5.0 inches
22	1.6-4.5 inches	4.2-5.0 inches	4.0-5.0 inches
23	2.5-4.5 inches New leaflets were seem to grown on 1 plant	4.3-5.0 inches	4.1-5.0 inches New leaflets were grown On 4 plants
24	2.5-4.7 inches	4.3-5 inches	4.5-5.3 inches
25	3.0-4.8 inches	4.5-5 inches	4.5-5.3inches
26	3.5-4.8 inches Leafs grown Bigger in size	4.5-5 inches	4.9-5.3 inches
27	3.5-4.8 inches Leafs grown Bigger in size	4.5-5.1 inches	5-5.5 inches
28	3.7-4.8 inches Leaflets observe on four plant	4.7-5.2 inches new leaflets observed on single plant	5.4-6.1 inches Leaflets observed on every plant

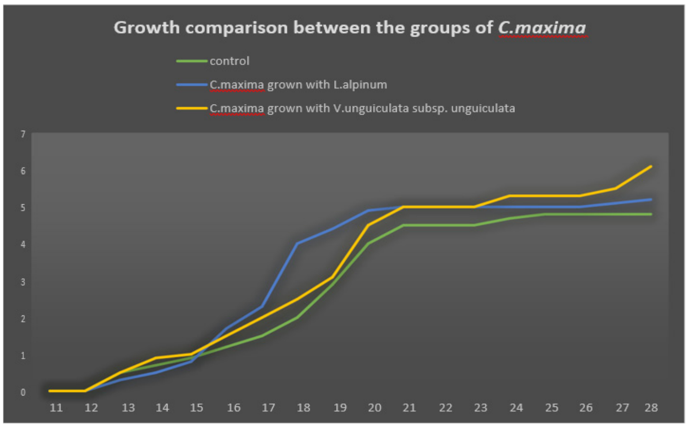

Figure 10: Growth comparison between different groups of *C. maxima.*

In graph, the x-axis shows the length of plants while the y-axis shows the days. Growth analysis curve of *C.maxima* when compared with control group shows maximum results with V.*unguiculata* subsp. *unguiculata* then a moderate result was seen with *L.alpinum* which is also a legume but its nitrogen fixing capability is not so efficient (Table 8 and Figure 10).

Table 9: Mean growth observation of *S. lycopersicum* groups.

Days	Control (solely)	Control with *L.alpinum*	Treatment With *V.unguiculata subsp. unguiculata*
11	Nothing observed	NO	NO
13	NO	NO	NO
15	6 seeds sprout	2 seeds sprout	4 seeds sprout
17	3 more seeds sprout Length 0.5-1.0 inches	2 more seeds sprout Length 0.7-1.5 inches	3 more seeds sprout Length 0.7-1.0 inches
19	Length 0.9-2 inches	Length 1.1-2 inches	1.0-2 inches
21	1.5-2 inches	1.7-2 inches	1.6-2 inches
23	1.9-2 inches	2 inches	1.8-2 inches
25	2 inches	2 inches	2 inches
27	2 inches	2 inches	2 inches
28	2 inches	2 inches	2 inches

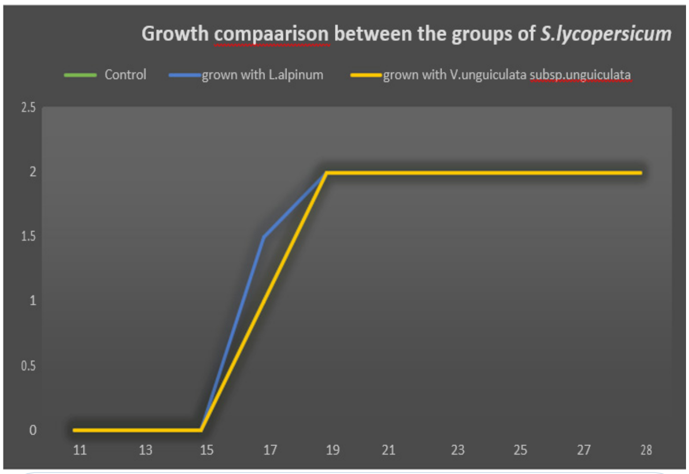

Figure 11: Growth comparison between different groups of *S. lycopersicum*.

In graph the x-axis shows the length of plants while the y-axis shows the days. The growth curve for S.lycopersicum shows no positive results when intercropped with legume, compared to control (Table 9 and Figure 11).

Chlorophyll content

A calculation and a comparison between the total chlorophyll content of different groups of *C.maxima* are as follows:

Table 10: Comparison between the chlorophyll content of *C. maxima*.

Groups	Control	With *L.alpinum*	With V.unguiculata subsp. unguiculata
Mean of total chlorophyll content	1.7975	4.629	6.65412

Chlorophyll is a photosynthetic pigment which determines a plant growth and its photosynthetic capacity, also closely related to plant nutrient status specifically plant nitrogen concentration in leaves because of which many researchers focused on estimating chlorophyll content when dealing with nitrogen fertilizers or when investigating the role of nitrogen on plant growth greater nitrogen concentrations means greater chlorophyll content. In Table 10 chlorophyll content seems to increased when intercropped with both legumes this means that legume contributed nitrogen in soil which is then used by *C.maxima* and ultimately enhances chlorophyll as compared to control.

Table 11: Comparison between the chlorophyll content of *S. lycopersicum.*

Groups	Control	With *L.alpinum*	With V.unguiculata subsp. unguiculata
Mean of total chlorophyll content	5.56718	5.298	4.967

In this Table 11, chlorophyll content did not enhanced by intercropping S.lycopersicum with both legume specifically with V.*unguiculata* subsp. *unguiculata* this is because of the competitive behavior, and negative uptake of nitrogen by S.lycopersicum during early growth.

Conclusion

Hence it is concluded that leguminous plants can itself enhance the growth of other non-leguminous crops, all these crops which were used to enhanced the productivity of other crops are legumes but they have different impacts on the same crop i.e. *Vigna unguiculata subsp. unguiculata* have a positive effect on the growth of Maxima cucurbita but it behaves neutral when grown with *Solanum lycopersicum*, it may due to the differences in the abilities of both these crops to take nitrogen from a legume, or differences in the behavior of both these crops with a legume, so if we want to reduce the use of chemical fertilizers, to sustains our ecosystem and go towards using natural components of ecosystem for our use (i.e. legume plants), we should find out the impacts of different legume when grow with other non-legume crops, and by using the best legume crop with a specific non-legume we can go for enhancing it yield without using chemical fertilizers.

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