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Efficacy of Neem (*Azadirachta indica* A. Juss) and Bael (*Aegle marmelos* (L.) Correa) Leaf Extracts as Biofertilizers in Cultivation of Mung Plant (*Vigna radiata* (L.) Wilczek)

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ABSTRACT

Biofertilizers are the need of the modern conventional agriculture and have given promising results in past studies while adding significant positive impact on soil. Our work was based on finding a new biofertilizer through different blends of extracts derived from leaves of neem and bael and to study its efficacy in the cultivation of mung plant. The analysis of biochemical parameters of leaves of mung plant suggested that the blend of neem : bael leaf extracts in the ratio of 1 : 2 produced the best results for the total chlorophyll content (0.723 mg/g), carotenoid content (0.098 mg/g), carbohydrate content (1.98 mg/g), amino acid content (13.26 µg/g) and protein content (0.53 mg/g). The soil samples from the same setup also gave highest values for the enzymatic activities with respect to invertase (7.62 µg glucose/g dry wt./h) and protease (0.266 mg tyrosine/g dry wt./h). The other blends of the leaf extracts also showed a significant higher value with respect to the biochemical parameters of leaves, physico-chemical properties and enzymatic activities of soil as compared to the control setup. A significant positive correlation between the invertase and protease activities (r = 0.952) of soil derived from the different blends suggests the same. This highlights the fact that the extracts derived from the leaves of neem, bael and their blends have good potential to serve as biofertilizers.

INTRODUCTION

Agriculture is the backbone of Indian economy. With a major portion of Indian population depending directly on agriculture, the stress over a small land resource to sustain a huge population is intense. Synthetic fertilizers were once considered to be a useful alternative to combat this issue. However, owing to its long term detrimental effects on soil (Adediran & Banjoko 2003, Osundare 2004), the synthetic fertilizers are not recommended by many scientists today (Rajasekaran 2012).

Biofertilizers represent any substance from manure to plant extract that can be expected to increase the productivity of crops simultaneously retaining the health and enhancing the sustainability of soil. Substances derived from plants that have the potential to give a greater crop yield while maintaining the soil quality for a longer run is an area of research interest have been developed through the concept of sustainable agriculture in Tropical Soil Biology Fertility (TSBF) program. Substances derived from plant origin containing living microorganisms having the capacity to replace synthetic fertilizers have been much studied in recent times. However, plant extracts have not received much attention as Biofertilizers and have been only studied for their pest control ability.

Neem (*Azadirachta indica* A. Juss), an indigenous plant species, is very often found to have a good pesticidal effect due to the presence of a major bioactive isomer, azadirachtin (Gahukar 2011). However, Lokanadhan et al. (2012) reported that the addition of neem extracts to soil has not only restricted pest infestations but also has improved the crop yield and enhanced various nutritional status of soil.

Bael (*Aegle marmelos* (L.) Correa) is a traditional plant, known for its rich medicinal value, contains many bioactive molecules like β-sitosterol, aegelin, lupeol, rutin etc. (Patel et al. 2012). Besides having antifungal and antibacterial properties, bael has also been reported to contain varied classes of compounds of alkaloids, fatty acids, amino acids etc. (Neeraj et al. 2017), which when subjected to decomposition, can provide essential nutrients to soil for plant uptake assisting in productivity.

In the quest of search of a new Biofertilizer, the present research aimed to study the efficacy of the leaf extracts of neem and bael as Biofertilizers in cultivation of mung crop. This study also made an effort to evaluate the biological property of soil through its enzymatic activities and assess the fertility status of soil.

MATERIALS AND METHODS

Preparation of the plot: The experiment was conducted in the red and lateritic soil of Sambalpur, Odisha (located in the eastern plateau and hilly agroclimatic zone of India). Mung bean (*Vigna radiata* (L.) Wilczek) seeds were obtained from local market and soaked in distilled water for 24 hours. The experimental area, prepared for the study, was divided into six plots each measuring 75 cm × 50 cm, and protected from all sides by mounts of soil to guard any surrounding entry of water or organic matter. Fifty seeds of mung bean were sown in each plot. During the period of the experiment, watering was done based on plant requirements. 150 mL of the five blends of leaf extracts (only neem, only bael, neem : bael = 1 : 1, neem : bael = 2 : 1 and neem : bael = 1 : 2) were applied to five plots separately and the sixth plot was left for control setup. Then leaf samples of the mung plant from various setups including control were collected on 7^{th}, 14^{th} and 21^{st} day for the analysis of the biochemical parameters while the soil samples from the respective setups were collected on 0^{th}, 7^{th}, 14^{th} and 21^{st} day for the analysis of physico-chemical parameters and enzymatic activities.

Preparation of leaf extracts: 100 g of green neem and bael leaves were taken. These leaves were then soaked overnight in distilled water. The next day, they were ground separately with 500 mL of distilled water each and the extract was then filtered and kept in refrigerator for further use.

Biochemical analysis of leaf: The pigment (i.e. total chlorophyll and carotenoid) content of the leaves was analysed by the acetone method given by Arnon (1949), while the carbohydrate content of the leaves was analysed following the anthrone reagent method (Yemm & Wills 1954). Similarly, the amino acid content of the leaves was determined by the ninhydrin method given by Moore & Stein (1948), and the protein content of the leaves by the folin's reagent method (Lowry et al. 1951).

Physico-chemical parameters and enzymatic activities of soil: Soil pH and soil conductivity were the two physico-chemical parameters determined in the present study following the methods mentioned by Sahu et al. (2016a), whereas the estimation of protease activity was done by sodium caesinate method (Ladd & Jackson 1982). The estimation of invertase activity was performed by Sorenson's buffer method (Ross 1983).

RESULTS AND DISCUSSION

Results: The present study evaluated the biochemical parameters of leaf samples of mung plant, the physico-chemical parameters and enzymatic activity of soil subjected to different treatments with Biofertilizers (leaves extract of neem and bael). It was found that there was a general progressive significant increase in all the biochemical parameters of leaf samples, physico-chemical parameters and enzymatic activities of soil under all the treatments as well as control setup with respect to days.

The data for different biochemical parameters of leaves, physico-chemical properties and enzymatic activities of soil under various treatments of neem and bael leaf extracts are presented in Table 1. The highest value for chlorophyll content in leaves of mung plant among various treatments in setups L1 (only neem), L2 (only bael), L3 (Neem : Bael = 1 : 1), L4 (Neem : Bael = 2 : 1) and L5 (Neem : Bael = 1 : 2) were found to be 0.458, 0.193, 0.483, 0.232 and 0.723 mg/g respectively, while the lowest value of chlorophyll content in the above setups was found to be 0.116, 0.052, 0.128, 0.085 and 0.263 mg/g respectively. Similarly, the highest carotenoid content in leaves of the treatments of L1, L2, L3, L4 and L5 was found to be 0.005, 0.003, 0.007, 0.005 and 0.010 mg/g respectively, while that of the lowest carotenoid content in the same setups was found to be 0.003, 0.001, 0.003, 0.003 and 0.003 mg/g respectively. In the control setup (L6), the values of chlorophyll and carotenoid content in the leaves of mung plant ranged from 0.049-0.098 and 0.001-0.003 mg/g respectively. Significant changes with respect to days and treatments were observed in both chlorophyll ($F_1 = 12.30$, $F_2 = 6.01$; $p < 0.05$) and carotenoid ($F_1 = 13.00$, $F_2 = 9.26$; $p < 0.05$) contents.

The maximum carbohydrate content among all the treatments in L1, L2, L3, L4 and L5 setups were found to be 0.65, 0.31, 1.47, 0.34 and 1.98 mg/g respectively, while that of the lowest values of carbohydrates in the same setups to be 0.09, 0.08, 0.07, 0.08 and 0.44 mg/g respectively. The control setup (L6), however, showed a value of carbohydrate content in the range of 0.05-0.27 mg/g. The carbohydrate content varied significantly only with respect to days ($F_1 = 6.22$; $p < 0.05$).

The amino acid content in leaves of all the treatments of L1, L2, L3, L4 and L5 ranged from 4.55-7.56, 1.54-1.78, 2.67-4.43, 2.44-3.79 and 6.66-13.26 µg/g respectively. Similarly, the control setup (L6) showed an amino acid content in the range of 2.32-3.48 µg/g. Significant variation was observed both with respect to days and treatments with respect to amino acid content ($F_1 = 4.98$, $F_2 = 18.44$; $p < 0.05$).

The protein content of leaves of mung plant, subjected to various treatments in L1, L2, L3, L4 and L5 were found to be in the range of 0.024 -0.048, 0.019-0.030, 0.023-0.043, 0.022-0.039 and 0.024- 0.053 mg/g respectively. The control setup (L6) at the same time showed a value of 0.022-0.034 mg/g of protein content. When analysed for statistical significance test with respect to days and treatments,

Table 1: Biochemical parameters of leaves, physico-chemical properties and enzymatic activities of soil under various treatments of neem and bael leaf extracts.

Parameters	Days	L1	L2	L3	L4	L5	L6
Total chlorophyll (mg/g)	7th	0.116	0.052	0.128	0.085	0.263	0.049
	14th	0.126	0.054	0.239	0.153	0.269	0.050
	21st	0.458	0.193	0.483	0.232	0.723	0.098
Carotenoid (mg/g)	7th	0.003	0.001	0.003	0.003	0.003	0.001
	14th	0.004	0.002	0.005	0.004	0.008	0.002
	21st	0.005	0.003	0.007	0.005	0.010	0.003
Carbohydrate (mg/g)	7th	0.09	0.08	0.07	0.08	0.44	0.05
	14th	0.39	0.23	0.40	0.32	0.55	0.15
	21st	0.65	0.31	1.47	0.34	1.98	0.27
Amino acids (µg/g)	7th	4.55	1.54	2.67	2.44	6.66	2.32
	14th	4.84	1.68	4.16	3.61	12.08	2.57
	21st	7.56	1.78	4.43	3.79	13.26	3.48
Protein (mg/g)	7th	0.024	0.019	0.023	0.022	0.024	0.022
	14th	0.038	0.026	0.037	0.035	0.041	0.031
	21st	0.048	0.030	0.043	0.039	0.053	0.034
Soil pH	0th	6.30	6.56	6.54	6.71	6.95	6.87
	7th	6.40	6.59	6.60	6.75	6.96	6.89
	14th	6.83	6.96	6.65	6.89	6.97	6.91
	21st	6.85	6.98	6.75	6.91	6.98	6.94
Soil conductivity (µS/cm)	0th	151.1	160.3	173.6	140.7	168.4	197.7
	7th	170.5	182.3	180.9	185.9	175.4	208.7
	14th	208.6	189.5	190.1	198.0	183.9	211.5
	21st	211.2	190.4	208.8	200.1	191.9	212.4
Protease (mg tyrosine/g dry wt./h)	0th	0.158	0.109	0.181	0.152	0.191	0.132
	7th	0.209	0.131	0.220	0.167	0.255	0.156
	14th	0.227	0.160	0.244	0.204	0.257	0.172
	21st	0.241	0.187	0.255	0.235	0.266	0.177
Invertase (ìg glucose/g dry wt./h)	0th	2.25	0.37	3.28	0.72	3.64	0.36
	7th	2.95	1.31	3.38	1.66	4.59	0.69
	14th	3.23	1.34	4.13	2.23	6.11	0.69
	21st	3.44	1.72	4.31	3.09	7.62	0.99

L1 = Neem, L2 = Bael, L3 = Neem : Bael = 1 : 1, L4 = Neem : Bael = 2 : 1, L5 = Neem : Bael= 1 : 2, L6= Control

significant variation was found both between days and between treatments with respect to protein content in the leaves of mung plant ($F_1 = 43.61$, $F_2 = 6.48$; $p < 0.05$).

It is evident from Table 1 that the soil pH of all the treatments including the control setup was found in the near acidic range. The soil pH of setups L1, L2, L3, L4, L5 and L6 (control) were found to be in the range of 6.30-6.85, 6.56-6.98, 6.54-6.75, 6.71-6.91, 6.95-6.98 and 6.87- 6.94 respectively. Significant variation was found both with respect to days and treatments ($F_1 = 7.11$, $F_2 = 6.70$; $p < 0.05$). Similarly, a gradual increase in the soil conductivity was seen with the change in days and the highest value was found to be 211.2 µS/cm, and the lowest to be 151.1 µS/cm (both the values recorded in L1 setup) among all the treatments, while in the control setup, the highest and lowest values for soil conductivity were found to be 212.4 and 197.7 µS/cm respectively. Significant changes were observed both with respect to days and treatments ($F_1 = 14.18$, $F_2 = 3.83$; $p < 0.05$).

The treatments in various setups of L1, L2, L3, L4, L5 and L6 showed the protease activity (mg tyrosine/g dry wt./h) between the range of 0.158-0.241, 0.109-0.187, 0.181- 0.255, 0.152-0.235, 0.191-0.266 and 0.132-0.177 respectively. The statistical significance test suggested that the protease activity in soils was significantly different both with respect to days and treatments ($F_1 = 51.30$, $F_2 = 47.88$; $p < 0.05$).

The treatments in various setups of L1, L2, L3, L4, L5 and L6 showed the invertase activity (µg glucose/g dry wt./h) between the range of 2.25-3.44, 0.37-1.72, 3.28-4.31, 0.72 - 3.09, 3.64-7.62 and 0.36-0.99 respectively. The statistical significance test suggested that the invertase activity in soils was significantly different both with respect to days and treatments ($F_1 = 10.49$, $F_2 = 39.75$; $p < 0.05$).

A correlation matrix taking all the soil parameters irrespective of treatments was worked out and presented in Table 3. An asterisk (*) marked value in the table represents strong positive or negative correlation at 0.05 level of significance. It is evident from the table that a significant positive correlation was found between invertase activity and

Table 2: Two way ANOVA for various parameters between days and different treatments.

Parameters	*Source of Variation*	*SS*	*df*	*MS*	*F Cal*$_{(0.05)}$	*F Tab*$_{(0.05)}$	*S/NS*
Total Chlorophyll	Between Days	0.2114	2	0.1057	12.30	4.10	S
	Between Treatment	0.2582	5	0.0516	6.01	3.33	S
Carotenoid	Between Days	0.00003	2	0.00002	13.00	4.10	S
	Between Treatment	0.00005	5	0.00001	9.26	3.33	S
Carbohydrate	Between Days	1.56	2	0.78	6.22	4.10	S
	Between Treatment	1.54	5	0.31	2.46	3.33	NS
Amino acid	Between Days	16.94	2	8.47	4.98	4.10	S
	Between Treatment	156.72	5	31.34	18.44	3.33	S
Protein	Between Days	0.0011	2	0.0005	43.61	4.10	S
	Between Treatment	0.0004	5	0.0001	6.48	3.33	S
Soil pH	Between Days	0.27	3	0.09	7.11	3.29	S
	Between Treatment	0.42	5	0.08	6.70	2.90	S
Soil conductivity	Between Days	4907.85	3	1635.95	14.18	3.29	S
	Between Treatment	2210.79	5	442.16	3.83	2.90	S
Protease	Between Days	0.02	3	0.01	51.30	3.29	S
	Between Treatment	0.03	5	0.01	47.88	2.90	S
Invertase	Between Days	10.11	3	3.37	10.49	3.29	S
	Between Treatment	63.89	5	12.78	39.75	2.90	S

Table 3: Correlation matrix between various soil parameters in different treatments.

	Invertase	Protease	pH	Conductivity
Invertase	1.000			
Protease	*0.952	1.000		
pH	0.037	-0.087	1.000	
Conductivity	-0.491	-0.364	0.152	1.000

'*' $p < 0.05$

protease activity of the soil ($r \geq +0.812$; $p < 0.05$). It was also found that the soil pH exhibited an insignificant positive correlation with invertase activity and soil conductivity ($r \leq +0.812$; $p > 0.05$). Similarly both the enzymes (protease and invertase) showed insignificant negative correlation with soil conductivity ($r \leq -0.812$; $p > 0.05$).

Discussion: The determination of biochemical parameters of leaf samples gives a clear idea about the mineral uptake and potential of survival of a plant in an agricultural soil. Moreover, the determination of physico-chemical and enzymatic activities of soil suggests the soil health condition and can give a reflection of the soil fertility status. The integrated study of these aspects i.e., the biochemical parameters of the leaves of crop plant and the biological analysis of the cultivated soil where these crops are grown can reflect the actual fertility level of the soil under study. A test of these parameters after application of natural leaf extracts and their blends can suggest the potential of the extracts as biofertilizer.

Chlorophyll is the basic molecule responsible for the food preparation in plants, and high chlorophyll content in plants can actually direct the synthesis of more reserve food material in plants and hence responsible for an increase in biomass. The present study found the lowest value of chlorophyll content in the control plant than the other treatments and the level of chlorophyll content varied significantly among the various treatments. The lowest value of chlorophyll, found in the control setup, might have been due to the salinity level of the soil. High salinity tends to cause acute decrease of chlorophyll content (Seth et al. 2014).

Carbohydrate and especially starch is the reserve food material of plants, which supplies the all important fuels for its activity. It was observed from the study that the carbohydrate content was highest for the L5 setup, and lowest for the L6 setup i.e., the control setup. However, no significant variation with respect to the treatments was observed in carbohydrate content, which might have been due to the low photosynthetic activity (because of the young stage of plants) resulting in low starch synthesis rate. Moreover, starch content has been reported to be less in organic fertilizers than their synthetic counterparts (Strimumar & Ockerman 1990).

The highest concentration of protein and amino acid occurred in the L5 (neem : bael = 1 : 2) setup whereas the lowest concentration was observed in L2 (only bael) setup. Higher nitrogen activity has been reported under low carbohydrate production which could have been the main reason for protein and amino acid build up in the leaf tissue (Lazauskas & Razukas 2001). Another reason could have been the protease activity in soil, which is deemed to be the primary factor of nitrogen mineralization that could have

caused high nitrogenous ions to get released into the soil which could possibly have been taken up by the plants. This also suggests that the leaf extracts reflect a potential of nitrogen fixation that might have been responsible for the formation of nitrogen related compounds in plants. Substances capable of nitrogen fixation can serve as good Biofertilizers (Roychowdhury et al. 2014).

Soil pH plays an important role in nutrient cycling and microbial activities in soil because all the microorganisms function within a definite pH range. The plants also uptake nutrients in a given pH range only. In the present study, the soil samples were found to be in the near acidic range. However, there was a gradual increase in the pH with respect to days. The significant variation in soil pH with respect to treatments suggests that the by-products released during the assimilation of the chemical components of neem and bael might have increased the pH level of soil and supplied a suitable environment for the growth of plants.

Electrical conductivity is a measure of the salt content in the soil. The present study shows gradual increase in the electrical conductivity in all the soil samples. However, the EC was found to be highest in the control setup thereby suggesting comparatively high salt content in the soil. There was a significant variation of EC with respect to treatments, which suggests that the leaf extract blends helped in better mineralization of the nutrients.

Protease can exist both extra and intracellular. Protease catalyses the hydrolytic dissociation of proteins, converting them into chemically simpler unit of amino acids (Caldwell 2005). In the present study, the highest protease activity was observed in the soil of L5 setup (neem : bael = 1 : 2), whereas the lowest activity in L2 setup (only bael). Bael has been reported to have antibiotic and antifungal effect that might have reduced the microbial biomass level in the L2 setup and hence resulted in the least protease activity. Protease activity is high when the microbial biomass turnover rate is high (Danneberg et al. 1989).

Invertase plays an essential role in the carbon transformation and carbon cycle in soil, which hydrolyses soil carbohydrates to provide nutrients for plants and microorganisms. In the present study, the least activity of invertase was observed in the control setup, whereas the highest activity to be in the soil of L5 setup (neem : bael = 1 : 2). This may be due to the fact that the degradation of organic matter was high in the L5 setup. High organic matter decomposition is a result of high invertase activity (Sahu et al. 2016b).

Neem does not show a complete knock out effect in its pesticidal behaviour and tends to limit the pest population (Nishan & Subramanian 2014). Hence, the microbial biomass in the soil might have been restricted and not eliminated completely. On the contrary, the bioactive molecules present in bael have shown inhibitory and regulatory effects in previous studies (Das et al. 2012). Hence, it can be expected that these bioactive molecules in bael might have suppressed the bioactivity of neem that tends to act as an antimicrobial agent (Karki 2001, Mohanty et al. 2008) thereby providing a suitable micro-environment for the optimal enzymatic activity. This in turn might have resulted in the mineralization of organic matter in the soil, and hence, we find greater enzymatic activities in the L5 setup in both the cases (invertase and protease activities). This also suggests that the blends of the neem and bael leaf extracts might have resulted in the decomposition of organic matter and helped in the mineralization of soil. Substance possessing the ability of good mineralization of soil is very often considered as a good biofertilizer (Roychowdhury et al. 2014).

CONCLUSION

Biofertilizers have been the need of the present agricultural society for sustainable agricultural practices. It was expected that the leaf extracts from neem and bael plants having pesticidal effect may also behave as Biofertilizers because they promote the mineralization of organic matter to release nutrients, simultaneously supporting the biological activity in soil.

Our results support the above assumptions. All the blends of neem and bael leaf extracts produced better results than the control and the best result was obtained in the blend of L5 (neem : bael = 1 : 2). The present study has good scope for future research, like comparison of these blends with synthetic fertilizers and studies relating to the germination potential of various crops and their morphological analysis. The present study can only suggest that the blends of neem and bael leaf extracts show characteristics of a Biofertilizer and only the future works in this light can suggest whether it can be commercially affordable and agro-economically feasible for the farmers or not.

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