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INFLUENCE OF MODULIN (BIOSTIMULANT) ON GROWTH, YIELD AND GENE EXPRESSION OF CALMODULIN IN RICE UNDER LOWERED NPK FERTILIZERS

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Abstract

Rice (*Oryza sativa*), the prince among cereals is the premier food crop not only in India but world too (Chhabra, 2002). Long term fertilizer experiments revealed that neither chemical fertilizer nor organics alone can achieve sustainability in crop production. Modulin is a proprietary product of T. Stanes and Company Ltd. a foliar bio-stimulant for plants, with organic mineral activators, and presented in a soluble, easily absorbable and available form, is obtained through a fermentation process, from marine calcites. It is a suitable nutrient management system that is economic and eco-friendly for sustained productivity. The present field experiment was carried out at the Therkumangudi, Cuddalore district, to study the influence of Modulin as a foliar treatment on growth, yield and gene expression of calmodulin in rice along with lower NPK levels. The treatments comprised of T_1 -Control (100 % recommended dose of NPK (RDF) without Modulin); T_2- 75 % RDF + foliar applications of Modulin @ 1kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages, T_3 - 75 % RDF + foliar applications of Modulin @ 2 kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages, T_4- 100 % RDF + foliar applications of Modulin @ 1kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages and T_5- 100 % RDF + foliar applications of Modulin @ 2 kg ha^{-1} at vegetative stage, panicle initiation and, 5 % heads/spikes formation stages. The study revealed that the 100 per cent RDF + foliar applications of Modulin @ 2 kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages (T_5) significantly registered the highest values in growth and yield attributes. This treatment (T_5) recorded highest BCR of Rs.3.25 followed by other treatments. In addition, Modulin increased the genetic expression of calmodulin genes, which regulates the plant growth and development.

Key Words: Rice, Modulin, Foliar spray, Grain yield,

Introduction

Rice (*Oryza sativa* L.) is the main staple food crop of India, covering an area of about 46 million hectares, with production of more than 100 million tonnes, but the productivity level is very low (2.97 t ha^{-1}) and assures food security in India for more than half of the total population. To feed the exploding population, projection of India's rice production target for 2025 AD is 140 million tonnes, which can be achieved only by increasing the rice production by over 2.0 million tonnes per year in the coming decade. In contrast, recent slow down of yields in irrigated rice based cropping systems was noticed as result of deterioration of soil health and decline in productivity level (Dubey *et al.*, 2014). Excessive use of inputs, such as chemical fertilizers and pesticides in the intensive rice bowls, will likely result in resource degradation and environmental pollution will have adverse effects on human health. Simultaneous losses of vital biotic and abiotic ecosystem structures and functions will affect the environmental sustainability of the rice land. Continuous imbalanced use of fertilizers, leads to decline or stagnation in productivity, due to limitation of one or more nutrients. The escalated costs and pollution hazards to agro-ecological situations, due to use of fertilizers are the other factors that weigh in favour of integrated nutrient management. The results of long-term experiments revealed that sustainable production could be achieved only by maintaining a balance between supply and demand of nutrients by integration of fertilizers.

The Modulin is a proprietary product of T. Stanes and Company Ltd., and a foliar bio-stimulant for plants, with organic mineral activators in a soluble, easily absorbable and available form. It stimulates key genes which are multifunctional and play an important role in the metabolic regulation, enabling the plant to respond to signals and modulates its activity to a wide range of downstream proteins implicated in diverse physiological processes.

Modulin works as a signal inducer, to the plant to respond, activate its metabolism and ensure higher levels of leaf absorption rates. It is nontoxic and environmentally safe. It is a foliar bio-stimulant derived by employing a unique manufacturing process and a superior foliar signal transducer, and is obtained through a fermentation process. Considering the above facts, the present study were carried out to study, the effect of Modulin foliar spray on rice growth, yield and qualities characteristics, under lower NPK levels and assess its effect on the expression of a key gene in the plant.

Materials and Methods

The field experiment was conducted in farmer's field located at Therkumangudi, Cuddalore district, Tamil Nadu. The field is situated at 11°33' North latitude and 79°69' East longitude at an altitude of +9.00 m above mean sea level. The crop rice was raised during *samba* season of 2017. For the treatments T_1, T_4, T_5, recommended dose of inorganic fertilizer nutrients *viz.,* nitrogen @150 kg ha^{-1} through urea, phosphorus @50kg ha^{-1} through single superphosphate and potash @50kg ha^{-1} through muriate of potash were applied. For the treatments T_2 and T_3 75 per cent recommended dose of inorganic fertilizer nutrients were applied. Besides, foliar application of Modulin at specified doses of 1kg and 2kg ha^{-1} were taken up at different growth stages *viz.*, vegetative stage, panicle initiation and 5% heads/spikes formation stages. The experiment was laid out in Randomized Block Design (RBD) with five replications. The treatments comprised of T_1 - Control (100% recommended dose of NPK (RDF) without Modulin), T_2 - 75% RDF + foliar applications of Modulin @1kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/ spikes formation stages, T_3 - 75% RDF + foliar applications of Modulin @2kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/ spikes formation stages, T_4 - 100% RDF + foliar applications of Modulin @1kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/ spikes formation stages and T_5 - 100% RDF + foliar applications of Modulin @2kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/spikes formation stages.

Ten hills in rice was chosen at random within each net plot and tagged for recording biometric observations at various stages as described below. Plant height was recorded from ground level to the tip of top most leaf $panicle^{-1}$ for rice at tillering, flowering and at harvest and expressed in cm. The number of tillers produced m^{-2} was recorded at maximum tillering stage. Five plants were removed at random from each treatment plot without damaging the roots and washed. The samples were sun dried initially for 24 hrs and subsequently oven dried at 80ºC to attain a constant weight. Then the DMP was recorded at harvest and expressed in t ha^{-1}. The panicles were randomly chosen for recording the number of grains $panicle^{-1}$. The total number of grains panicle $^{-1}$ were counted and recorded. The panicles were randomly chosen for recording the panicle length. The length of panicles is measured in cm from the base to the tip of the panicle. Thousand filled grains were counted from the bulk of grains drawn at random in each plot and weighed at 14 percent moisture content and expressed in g. The matured crop was harvested from the net plot area, the grains were separated by hand threshing, winnowed, and sun dried sufficiently. The dried grains were weighed at 14 percent moisture level and recorded plot wise and computed and expressed in kg ha^{-1}. Nitrogen Use Efficiency (NUE) is a term used to indicate the ratio between the amount of grain yield in kg ha^{-1} to the amount of fertilizer N applied (Novoaand Loomis, 1981).

Biochemical Analysis - Enzyme Extraction and Assay

The leaf samples, weighing about 200mg, were homogenized with 10 ml of phosphate buffer pH 6.8 (0.1 M) and divided into two equal 5-mI portions. One 5-ml portion was centrifuged at 2°C for 15 min at 17,000g in a refrigerated centrifuge. The clear supernatant was taken as the enzyme source.

The activity of catalase as well as peroxidase was assayed after the method of Chance and Maehly (1955) with the following modifications. Five milliliters of the assay mixture for the catalase activity comprised: 300 u moles of phosphate buffer, pH 6.8, 100 μmoles of H_20_2, and 1 ml of the twice diluted enzyme extracted. After incubation at 25°C for I min, the reaction was stopped by adding 10 ml of 2% (v/v) H_2SO_4 and the residual H_20_2 was titrated against 0.01 N $KMnO_4$ until a faint purple colour persisted for at least 15 sec. A control was run at the same time in which the enzyme activity was stopped at "zero" time. One unit of catalase activity is defined as that amount of enzyme which breaks down 1 μmol of

H_2O_2/min under the assay conditions described. For Peroxidase analysis, Five millilitres of the assay mixture for the peroxidase activity comprised: 125 µmoles of phosphate buffer, pH 6.8, 50 µmoles of pyrogallol, 50 µmoles of H_2O_2, and 1 ml of the 20 times-diluted enzyme extract. This was incubated for 5 min at 25°C after which the reaction was stopped by adding 0.5 ml of 5% (v/v) H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbancy at 420 nm. The Nitrate reductase was estimated according to the method of Vega, JM, Jacobo, C and Mannual, L (1980) and the Protein content was estimated according to the method of Ali-khan and Youngs, (1973).

Expression analysis (qPCR studies)

RNA Extraction

The leaves from T_1 to T_5 were collected and fixed in the liquid nitrogen and transported to the laboratory in ice cold condition. The RNA was extracted using the RNA Express reagent according to the manufacturer's protocol. The extracted RNA was quantified using the Nanodrop lite. The quality of the RNA was checked using the agarose gel electrophoresis.

DNA Synthesis

One µg of total RNA was used to synthesize cDNA with 1st strand cDNA synthesis kit (Hi-cDNA Synthesis Kit) according to manufacturer's instructions. The authenticity of cDNA was confirmed using agarose gel electrophoresis and Nanodrop quantification.

Primer validation

Based on the previous report, the primers for *OsCam1* genes for rice were validated using real time PCR from the control cDNA samples. Amplification curves were monitored for 40 cycles. Melting curves were analyzed for single product formation in qPCR runs. The sequences and length of the primers used are given below.

RT-PCR:

Real Time (RT-PCR) was carried out by using Bio-Rad SYBR Green master mix; 10µl reaction volume for each sample was prepared in 96-well PCR plate (BioRad) according to the manufacturer's instruction. After preparing the reaction mixture RT PCR was carried out in BioRad Real-Time PCR. qPCR thermal cycling steps consisted of reverse transcription at 50°C for 15 min. followed by, initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 30 s, 60°C for 30s, 72°C for 1 min. The sigmoidal curves and relative normalized gene expression were obtained through qPCR.

The gross and net income hectare^{-1} for each treatment based on the prevailing market rates was worked out. The net income was calculated by deducting the cost of cultivation from the gross return. Return rupee^{-1} invested was worked out by dividing the gross return by the cost of cultivation**.** The data were statistically analyzed as per the method of Panse and Sukhatme (1985).

Details of primers used in this study.

Gene Name	Primer name	Primer Sequence	Amplicon size (bp)
Oryza sativa	*OsCam1*-1-F	ACCGTGCATTGCCGTATTAG	177
Cam 1	*OsCam1*-1-R	GCAAGCCTTAACAGATTCAC	

Results and Discussion

1. Growth parameters

Among the treatments, foliar application of Modulin exhibited significant influence on the varied growth attributes *viz*., plant height and dry matter production compared to the recommended dose of fertilizers. 100 per cent RDF + foliar applications of Modulin @2kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages (T_5) significantly registered the highest values in all these growth attributes (Table 1). The other treatments *viz.,* 100 percent RDF + foliar applications of Modulin @ 1kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages (T_4) and 75 percent RDF + foliar applications of Modulin @2 ha^{-1} at vegetative stage, panicle initiation and 5% heads/ spikes formation stages (T_3) which was on par with each other stood next in order of ranking. The treatment (T_1) 100 percent recommended dose of NPK (RDF) without foliar spray of Modulin (control) recorded the least values of growth attributes. From the perusal of experimental results, it is evident that the values of rice growth and yield components *viz.,* plant height, number of tillers m^{-2}, DMP, panicle length and grain yield were significantly higher with foliar application of Modulin at vegetative stage, panicle initiation and 5% heads/ spikes formation stages of rice. This increase in the growth parameters is attributed to the factor that in addition to major nutrients NPK, modulin is a good source of mineral activators and has an added advantage over the other sources of nutrients in terms of its solubility, bio availability and translocatability. The combination of various secondary nutrients with organic acids obtained through a fermentation process, served as a secondary messenger in assisting, various plant functions, from nutrient uptake to changes in cell status to help the plant react to the impact of environmental and disease stresses and plays a key role in the plant growth and development as a signaling molecule.

Foliar application of Modulin in rice, resulted in vigorous root, which could be through enhanced cell division, tissue differentiation and metabolism of nucleic acid; good shoot initiation reflecting upon enhanced crop growth and establishment in terms of plant height. Foliar application of Modulin in rice promoted higher nutrient uptake and utilization of plant nutrients for enhanced photosynthates production and crop canopy establishment. This has positively reflected upon crop dry matter production. Recommended NPK alone (T_1) resulted in the least values of growth parameters, attributable to the absence of beneficial effect of Modulin, a good source of organically solubilised marine calcites.

2. Yield parameters

The yield components also *viz.,* number of tillers m^{-2}, panicle length, number of grains $panicle^{-1}$ thousand grain weight and seed yield were highest with the 100 percent RDF + foliar applications of Modulin @2 kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/spikes formation stages (T_5). The treatment (T_1) 100 percent recommended dose of NPK (RDF) without foliar spray of Modulin (control) recorded the lowest values of these yield components (Table 1).

Yield is the manifestation of yield attributing characters (Matsushima, 1976) and higher grain yield was influenced mainly by yield attributing components like number of tillers m^{-2}, panicle length, filled and unfilled ratio. Significant increase in yield of rice due to foliar application modulin and the different levels of blanket practice, at vegetative stage, panicle initiation and 5% heads/spikes formation stages of rice could be further attributed to increased growth and vigour of plant as evident from increased yield attributes. Higher total dry matter production was due to the fact that crop nitrogen requirement was met throughout the crop growth. This in turn might have increased the yield attributing characters and yield of rice. Similar inferences were documented by Hossain *et al.,* (2010). Tillering is the outcome of the expansion of auxillary buds which is closely associated with the nutritional condition of the mother culm/tiller, as it receives carbohydrates and nutrients from the mother culm (Dash *et al.*, 2010) during its early growth period, and was observed to be improved with foliar application of modulin. Increased tiller production could be due to the greater supply of N with efficient utilization for cell multiplication, enlargement and

Table 1: Effect of foliar application of Modulin on growth and yield attributes of rice.

Treatments	Plant height at harvest(cm)	Number of tillers m^{-2}	Panicle length (cm)	No. of grains $panicle^{-1}$	dry matter production (kg ha^{-1})	Test weight (g)	Grain yield (kg ha^{-1})
T_1 -Control (100 % recommended dose of NPK (RDF) without Modulin	69.78	313	19.30	136.00	6400	18.68	4250
T_2- 75 % RDF + foliar applications of Modulin @ 1kg ha^{-1} at three stages	72.95	324	21.55	166.00	6700	18.70	4500
T_3 - 75 % RDF + foliar applications of Modulin @ 2 ha^{-1} at three stages	79.60	358	22.51	182.50	7250	18.72	4850
T_4- 100 % RDF + foliar applications of Modulin @ 1kg ha^{-1} at three stages	81.27	361	22.81	185.50	7300	18.73	5005
T_5- 100 % RDF + foliar applications of Modulin @ 2kg ha^{-1} at three stages	87.65	401	26.15	209.00	8200	18.76	5400
$S.E_D$	0.90	3.67	0.29	2.57	43.25	0.01	77.98
CD (p=0.05)	1.96	8.00	0.65	5.60	95.59	0.04	170.00

Table 2: Cost of cultivation

Treatments	Cost of cultiv-ation(Rs./ha)	Gross retu-rns(Rs./ha)	Net returns (Rs./ha)	B:C Ratio
T_1 -Control (100 % recommended dose of NPK (RDF) without Modulin	31,800	83,500	41,200	2.62
T_2- 75 % RDF + foliar applications of Modulin @ 1kg ha^{-1} at three stages	30,550	88,000	46,600	2.88
T_3 - 75 % RDF + foliar applications of Modulin @ 2 ha^{-1} at three stages	30,700	94,300	52,000	3.07
T_4- 100 % RDF + foliar applications of Modulin @ 1kg ha^{-1} at three stages	31,950	97,090	53,280	3.03
T_5- 100 % RDF + foliar applications of Modulin @ 2kg ha^{-1} at three stages	32,100	1,04,200	59,600	3.24

(Cost of modulin; 150 Rs./kg)

formation of nucleic acids and other vitally important organic compounds in the cell sap (Cardeans, 1990).

Economic yield is a complex inter-relationship of its components, which are determined from the growth rhythm in vegetative phase and its subsequent reflection in reproductive phase. Grain yield is the manifestation of yield attributing characters in rice (Nisar, 2000). It is observed from the trial data that 100 per cent RDF along with foliar application of Modulin (T_5) and other treatments brought about significant improvement in grain yield and established superiority over the treatment 100 per cent RDF alone without modulin (T_1) due to increased absorption of nutrients and their assimilation even under lowered application of NPK. Correlation studies have shown that grain yield is highly correlated with yield attributes. Increase in grains per panicle is attributed to foliar application of Modulin, which is responsible for augmentation of the physiological potential in plants and elicitation of plant responses by triggering the plants to respond efficiently to any developmental signals. This had the ultimate effect on translocation of synthesized food to grains. Modulin is a good source of organically

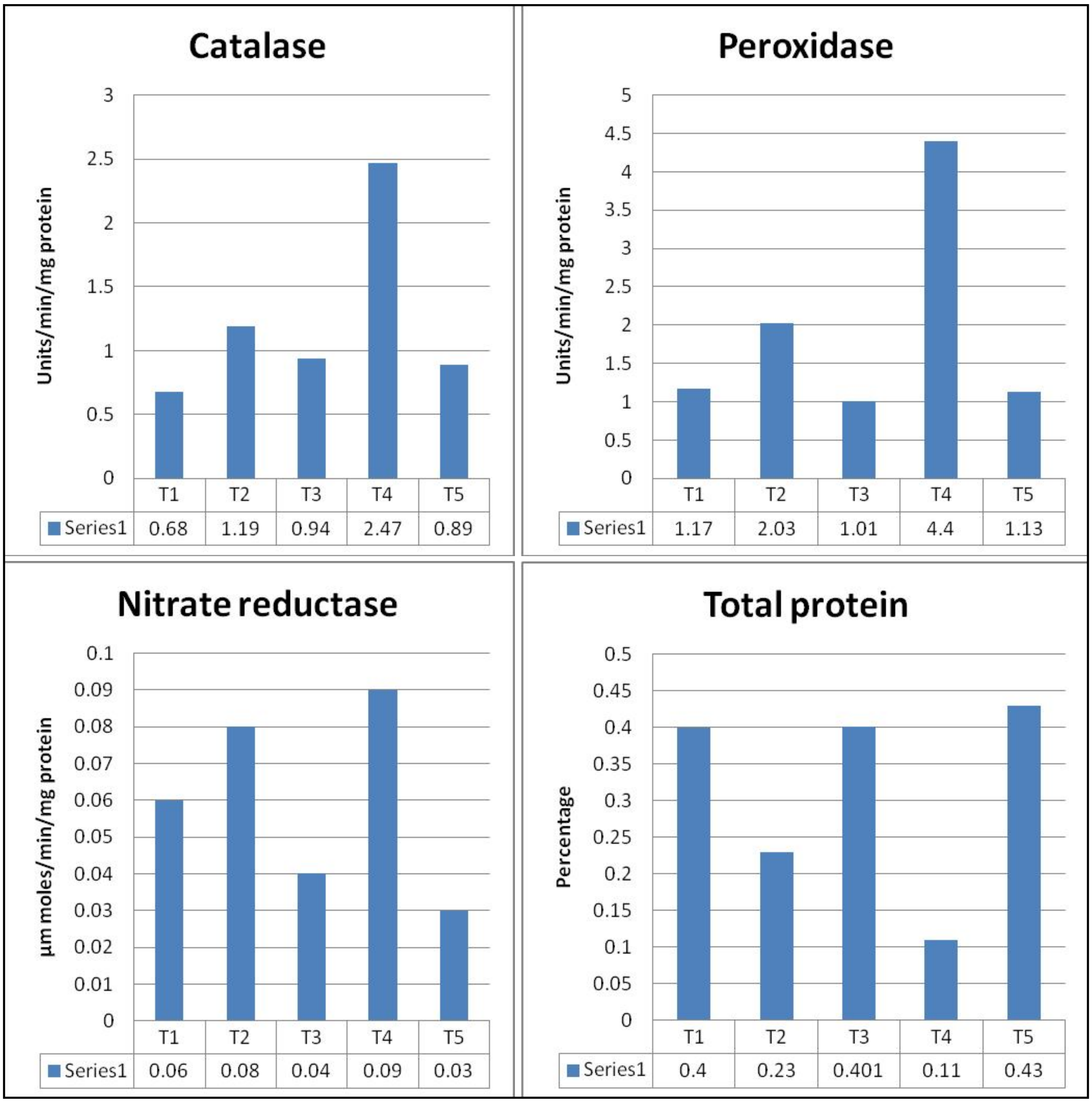

Fig. 1: Enzyme activities in test and control plants

complexed marine calcites with leaf protein concentrates and organic acids, and has an advantage in terms of its solubility, bioavailability and translocatability.

It is by and large, true that rice varieties have high rate of responsiveness towards fertilizer application and more particularly for N because of their conducive genetic makeup. In physiological terms, yield of most cereals is largely governed by source (photosynthesis) and sink (grain growth) relationship (Evans and Wardlaw, 1976). However, capacity of the metabolic system transporting the photosynthates and partitioning of assimilates between their sites of utilization *i.e.*, sink are the major determinants of crop yield (Gifford and Evans, 1981).

The treatment 100 percent RDF + foliar applications of Modulin @2kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/spikes formation stages (T_5) significantly registered the highest values in all growth and yield attributes. The other treatments *viz.,* 100 percent RDF + foliar applications of Modulin @1kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/spikes formation stages (T_4) and 75 percent RDF + foliar applications of Modulin @2kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/spikes formation stages (T_3) which was on par with each other stood next in order of ranking in all these parameters. The treatment 75 percent RDF + foliar applications of Modulin @1kg ha^{-1} at vegetative stage, panicle initiation and 5 % heads/ spikes formation stages (T_2) was next in order. It was also noted that there was a yield increase of 21 percent over the control in the treatment (T_5) and yield increase of 15.08, 12.38 and 5.55 percent respectively in (T_4), (T_3) and (T_2). But, in terms of nitrogen use efficiency, the treatment 75 percent RDF + foliar applications of Modulin @2kg ha^{-1} at vegetative stage, panicle initiation and 5% heads/spikes formation stages (T_3) recoded highest NUE of 43 followed by (T_2), (T_5) and (T_4). The least NUE of 28.3 per cent was observed in (T_1). The treatment (T_5) recorded highest BCR of Rs.3.25 followed by other treatments (Table. 2).

The activity of catalase, peroxidase, nitrate reductase and total protein was increased in T_4 followed by T_2 (Fig. 1), whereas, the activity of the enzymes in T_5 was lower than other group. The results revealed that the enzyme activity was decreased in the presence of Modulin with 100% RDF and the protein content in the T_5 and T_3 were higher than the G3, G2 and G1 (Fig. 1). These mineral activators, obtained through a fermentation process ensured, higher levels of leaf absorption rates. It contains organically complexed marine calcites with leaf protein concentrates, in a soluble and bio-available form, which serves as a secondary messenger, nutrient and mineral efficiency catalyst. The Calmodulin, Calmodulin-like proteins (CML), Calcineurin B-like proteins (CBLs), Ca^{+2} dependent protein kinases (CDPK's) elicited by developmental cues and environmental stresses mediate plant responses by regulating the transcriptional process.

3. Calmodulin gene expression

To confirm the expression of *OsCam1* genes in rice, we used control and test plants (T_1 – T_5) and performed real-time quantitative PCR (RT-qPCR). Convincingly, in T_5 and T_3 *CaM* gene expressed higher fold (Cq = 40.03 and 43.45) (Table 3 and Fig. 2) of expression, whereas, the other groups T1, T_2 and T_4 didn't show the Cq value. The expression level of the gene was highest in Test (T_5), when compared to the control (Table 3 and Fig. 2). Calmodulin is one of the best characterized, enzymes with no catalytic activity of its own but, activates numerous downstream target proteins. It has been shown that Calmodulin is involved in plant growth and development, and plant responses to environmental stimuli and stresses, such as osmotic stress, salinity, cold, heat, mechanical stress, oxidative stress, hormonal treatment, and pathogens. The activated Ca^{2+} – Calmodulin complex binds to target proteins and modulates

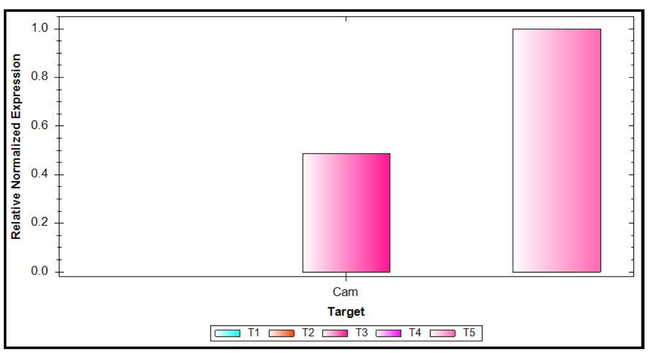

Fig. 2: Relative normalized gene expression of Calmodulin gene

Table 3: Cq values of Calmodulin and 18S rRNA gene.

Target	Sample	Ctrl	Expression	Expression SEM	Corrected Expression SEM	Mean Cq	Cq SEM
18S	T_2		N/A	N/A	N/A	18.60	0.00000
18S	T_3		N/A	N/A	N/A	18.08	0.00000
18S	T_4		N/A	N/A	N/A	18.75	0.00000
18S	T_5		N/A	N/A	N/A	15.70	0.00000
Cam	T_1		N/A	N/A	N/A	N/A	N/A
Cam	T_2		N/A	N/A	N/A	N/A	N/A
Cam	T_3		0.48543	0.00000	0.00000	43.45	0.00000
Cam	T_4		N/A	N/A	N/A	N/A	N/A
Cam	T_5		2.00000	0.00000	0.00000	30.03	0.00000

their activities, and finally results in physiological responses, such as cell growth or differentiation, stress tolerance or growth arrest, and cell death. Similarly, in the present investigation the total protein content of the T_5 was increased, indicates that it might activated some downstream target proteins resulting, in cell growth or differentiation.

Application of Modulin in rice showed considerable increase in the expression of calmodulin genes, thus modulating the genetic potential, physiological processes, organ development & yield in plants. Modulin treated rice crops T_3 and T_5 showed considerable fold of increase in the expression of calmodulin genes that influenced remarkably the yield. It infers that the expression of Calmodulin genes significantly increased the growth and yield of rice crops. Application of Modulin in rice showed considerable fold of increase in the expression of calmodulin genes that positively correlated with yield.

Hence, It may be inferred that foliar application of the product, Modulin from T. Stanes & Co. Ltd., is an eco friendly product and a stimulant for increasing growth and yield attributes in rice crop. This can be recommended as a foliar spray at vegetative panicle initiation and 5% heads/ spikes formation stages, along with recommended N, P and K or 75% and also at lower levels, holds promise as an eco-friendly and economically suitable nutrient management system, with emphasis to achieve the sustained production over a long period for rice.

Conclusion

Results of present investigation indicated that enough variability present in the population. Role of environmental factors were found very less in present study and the characters studied were reported predominantly influenced by genetic factors only. Higher value for both GCV and PCV were observed for number of effective tillers per plant, weight of panicle, number of filled grains per panicle, grain yield per plant, disease severity per cent and area under disease progress curve. High heritability with high genetic advance observed for number of filled grains per panicle, number of unfilled grains per panicle, spikelet fertility percent and area under disease progress curve which indicates involvement of additive gene effect and effectiveness of selection for these traits. Association studies revealed that number of effective tillers per plant, number of filled grains per panicle, plant height and spikelet fertility % positively corelated with grain yield per plant thus performing selection for these traits will be resulted into improvement of grain yield per plant. Leaf blast infection revealed causing significant decrease in grain yield per plant because both blast disease related traits in the study showed significant negative correlation with grain yield per plant. Significant negative correlation with both the blast related traits was also showed by plant height, test weight and panicle length, revealing that test weight will be decreased if blast disease present in the field as well as plant height and panicle length, respectively.

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