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# BIOSTIMULANT ACTIVITY OF PLANT PROTEIN HYDROLYSATE ON THE GROWTH OF *BETA VULGARIS SUBS. VULGARIS* (LINN.)

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## ABSTRACT

Natural biostimulants hold a huge promise for the future of agriculture. They assist the plant growth throughout the crop's life from seed germination to plant maturity. In this regard, natural biostimulants like protein hydrolysates are gaining more attention, since they can act as an alternative source for chemical fertilizers. Protein hydrolysates display hormone like function and influence plant metabolism by interacting with biochemical processes and physiological mechanisms, such as glycolysis and nitrogen assimilation. Wheat gluten hydrolysate a natural protein biostimulant has been known to have oligopeptides, polypeptides and free amino acid which can exhibit a hormone like activity resulting in an increased nitrogen uptake hence rendering its use as natural fertilizer supplement. In this study, the effect of wheat gluten hydrolysate, as biostimulant was studied under in vitro conditions to measure the shoot growth and also the gene expression of enzyme involved in the tricarboxylic acid cycle and nitrogen metabolism. An increased biostimulant activity of the wheat gluten hydrolysate would enable its application in the field of agriculture as a plant based fertilizer that would likely aim at reducing the use of chemical fertilizers and improving crop performance.

**KEYWORDS:** *Biostimulant, Fertilizer, Nitrogen metabolism, Protein Hydrolysate, tricarboxylic acid cycle, Wheat Gluten Hydrolysate*

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# INTRODUCTION

The application of biostimulants in crop cultivation allows higher levels of endurance by the reduction of fertilizers, environmental contamination and also increases plant tolerance to abiotic and biotic stresses[1-2]. Organic biostimulant compounds are classified into three major categories based on source and content: humic substances, seaweed extracts, and amino acids containing products[3-4]. Protein hydrolysates[5] (PHs) are mixtures of peptides and acids of animal or plant origin and amino acids such as glutamate, glutamine, proline and glycine betaine. They are synthesised by chemical, enzymatic or thermal hydrolysis[6-7]. They can act as plant growth regulators due to the presence of peptides and free amino acids. It is suggested that protein hydrolyzates may promote nitrogen assimilation in plants combined with regulation of C and N metabolism[8-9]. Biostimulants can be used as good production strategy for obtaining high yields of nutritional vegetables with lower impact on the environment[10-15]. The use of biostimulant had an effect on the morphological and phytochemical properties of plants in *invivo* and *invitro* conditions and on their mechanism of action[16-19]. A biostimulant based fertilizer (alfalfa and meat flour protein hydrolysate) was added as an supplement to the maize plants which increased the activities of nitrate reductase (NR) and malate dehydrogenase (MDH) , suggesting a positive role of the two protein hydrolysates in the induction of nitrate conversion into organic nitrogen. In addition, role of amino acids and small peptides of the two PHs is suggested in the regulation of the hormone-like activity and nitrogen pathway[20].Wheat gluten hydrolysate (WGH) is a natural biostimulant that has been analyzed to have oligopeptides, polypeptides and free amino acids which can elicit a hormone like activity resulting in an increased nitrogen uptake thus, rendering its use as a natural fertilizer supplement. They were also found to have a high relative abundance of stable glutamine. In this study, our aim is to analyze the biostimulant effect of the wheat gluten hydrolysate on the growth of *Beta vulgaris subs. vulgaris* (Linn.). The biostimulant activity of the wheat gluten hydrolysate was studied by measuring the growth parameters of the plant and studying the gene expression of the enzymes involved in the tricarboxylic acidcycle (TCA) and nitrogen metabolism through real time PCR, which are related to plant productivity.

# MATERIALS AND METHODS

***Invitro* culture of *Beta vulgaris subs. vulgaris* (Linn.)**

MS media was prepared for 20ml and autoclaved.1mg/L of wheat gluten hydrolysate was prepared as stock solution. The working solutions were prepared at different concentrations. The selected plant seeds were rinsed in 70% ethanol and were sterilized in 0.2% Mercuric chloride solution. The sterilized seeds were inoculated into the test tubes along with 50µL of the protein hydrolysate solutions and were stored for a period of 2-3 weeks. The shoot length of the *in vitro* grown proliferated plantlets was measured respectively.[21]

***RNA isolation and quantification***

About 100mg of the leaves from the proliferated plantlets were taken and 1ml of trizol was added to the cleaned leaves. The leaves were homogenised well and 10µl of β-mercaptoethanol was added to the mixture. The mixture was then centrifuged. To the supernatant, 200µl of chloroform was added and incubated at room temperature for 5 minutes, followed by the next centrifuge. After the phase separation, an equal amount of isopropanol was added to the mix and centrifuged. To the pellet formed, 300µl of ethanol was added and centrifuged for 5 minutes. The supernatant was discarded and left for air dry. The pellet was stored at -20ºC for further use. The integrity of the RNA (3µl of the RNA and 1µl of the dye was added) was checked using agarose gel electrophoresis. The sample was loaded in the gel electrophoresis. The bands were further viewed using the Bio Rad gel documentation instrument to visualize the 28S, 18S and 5S RNA bands.The quantification of the RNA was performed using a 96 well plate, in order to measure the purity and the concentration of total RNA. 150µl of DEPC water was used as the reference. The OD value was measured at 260 nm and 280 nm.[22]

***cDNA synthesis***

The cDNA was synthesized by using the cDNA synthesis kit, Takara.The mix was placed in the thermal cycler for 5 mins at 65ºC. Then it was allowed to cool in ice. To the same mix, 4µl of the RT Buffer, 0.5µl of RNase inhibitor, 1µl of Reverse Transcriptase were added and the mix was made up to a total volume of 20µl. Contents are mixed well and were placed in the thermal cycler.

***RT- PCR***

A volume of 11µl of SYBR Green Master Mix was taken in a PCR tube. Equal volumes of Primer Mix and cDNA were added. The Primer mix was a mixture of 0.5µl of the forward primer and 0.5µl of the reverse primer. To this 1µl of cDNA was added (Table 1). The contents were mixed and kept in the Real time PCR thermal cycler. The graphs and Cq values were analysed.

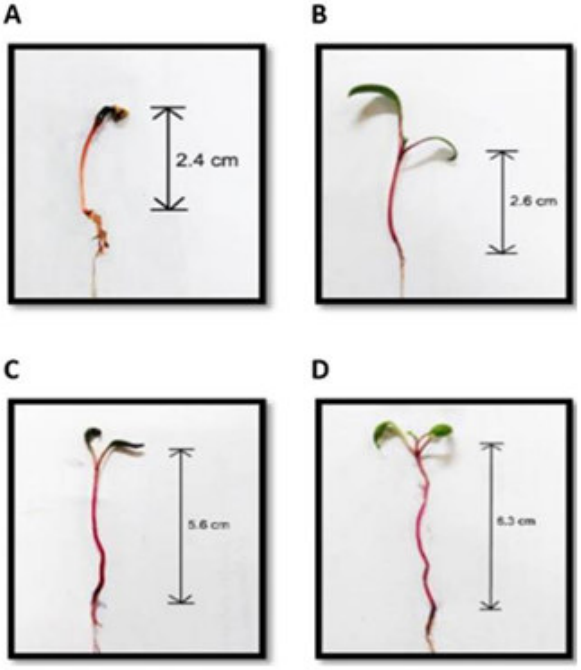
**Table 1**
***Oligonucleotide Primers of BetaVulgaris subs. vulgaris (Linn.) .***

| Gene | Forward Primer (5'- 3') | Reverse Primer (5'- 3') |
|---|---|---|
| Malate Dehydrogenase | TGCTCGATATTCCTCCTGCT | AGCAGATGCTTGGG ACTTGT |
| Nitrate reductase | GGACTGGACCGTTGAGGTAA | TGCTGATCCCCAGTTAAACC |
| Actin | CAGGGAAAAGATGA CCCAGA | ACGACCAGCAAGATCCAAAC |

## RESULTS

***Effect of wheat gluten hydrolysate on shoot growth of selected plantlets***

*Beta vulgaris subs. vulgaris (Linn.)* wascultured in *in vitro* conditions using three different concentrations- 0.1%, 0.01% and 1%, in order to study its effect on shoot growth. After a period of 2 weeks the seedlings grown in a protein hydrolysate medium showed increase in the length of the shoots which was found to be 2.6cm, 5.6cm, 6.3cm as compared with the control, which was found to be 2.4cm (Figure 1).

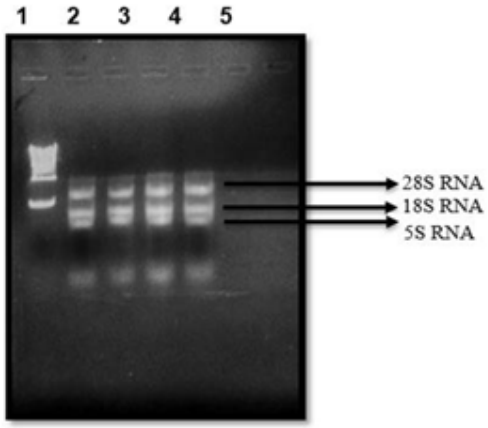



***A - Control(MS Media), B - WGH 0.01 mg/L, C - WGH 0.1 mg/L & D - WGH 1 mg/L***

**Figure 1**
***Measurement of shoot length of the proliferated Beta vulgaris subs. vulgaris (Linn.) plantlets.***

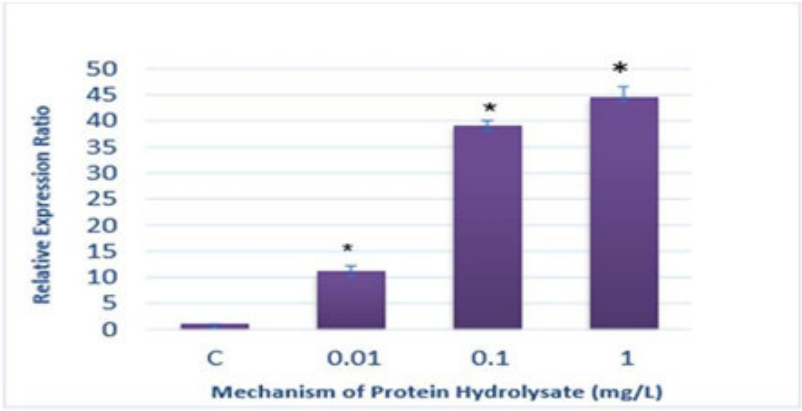
***Gene expression of TCA cycle and nitrogen metabolism enzymes***

The integrity of the RNA was observed from the isolated 28S, 18S and 5S RNA in the 1.2% agarosegel (Figure 2). The gene coding for enzyme malate dehydrogenase and nitrate reductase were analyzed using the q-PCR having the reference gene – Actin which played a role as the internal control. The results of agarose gel electrophoresis indicated a significant increase of mRNA abundance in the leaves of the plants treated with the protein hydrolysate as compared to the control plantlets. It was found that the induction of the genes was strongly dependent on the concentrations of protein hydrolysate where a gradual increase in expression was observed in the activity of the MDH gene as compared to the control (Graph 1). Similarly, significant increase in expression was observed in the NR gene in 1mg/L rather than at 0.01 and 0.1mg/L as compared to the control plants (Graph 2).



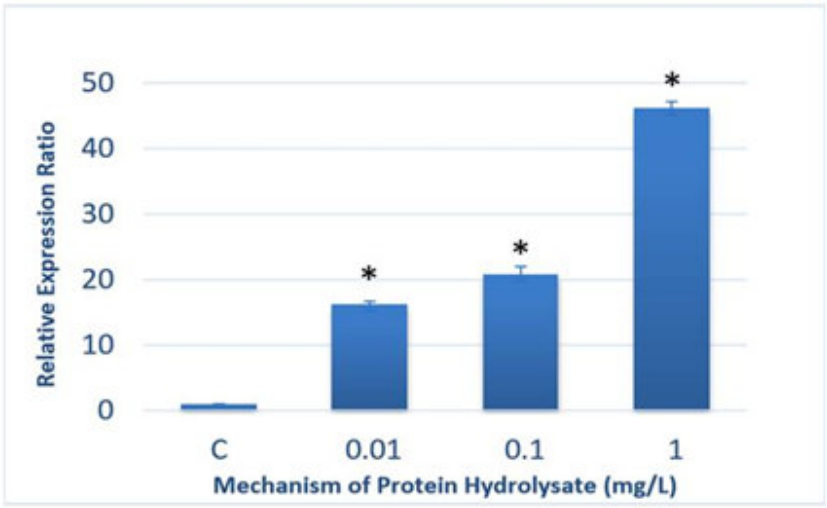

***1 - 1Kb RNA Ladder, 2 – Control, 3 - 0.01mg/L WGH, 4 - 0.1 mg/L WGH and 5 - 1 mg/L WGH***

**Figure 2**
***Agarose gel electrophoresis (1.2%) of the total RNA isolated from the leaves of Betavulgaris subs. vulgaris (Linn.)***




**Significant difference between treatments (P < 0.05)*

**Graph 1**
***Relative expression ratio of Malate dehydrogenase gene with respect to the Actin gene.***




**Significant difference between treatments (P < 0.05)*

**Graph 2**
***Relative expression ratio of Nitrate Reductase gene with respect to the Actin gene.***

## DISCUSSION

Our results are in line with other research findings who reported that the use of biostimulants had positive effect on total shoot length and biomass of the plantThe overall increase in the pigment content of leaves after biostimulant application was observed in pepper cultivars when compared with control plantlets[23-24]. Maize seedlings showed increased root and leaf growth which induced morphological changes in root architecture upon protein hydrolysate based fertilizer treatment[25-28].In this study, Wheat gluten hydrolysate acts as a biostimulant thereby inducing the up-regulation of the genes coding for MDH and Nitrate reductase enzymes as depicted in RT-PCR experiments. Similar results were obtained with maize seedlings supplemented with Alfalfa protein hydrolysate which improved the activity of malate dehydrogenase.[29] The activity of malate dehydrogenase (MDH) and oxaloacetate is required to synthesize aspartate which is produced from malate in the TCA cycle[30-31]. The results obtained in this study are in concordant with the data presented by Mesfin Tesfaye *et al*.[32] which depicted a 1.6-fold increase in malate dehydrogenase enzyme specific activity in root tips of selected transgenic alfalfa. Nitrate reductase (NR) plays a major role in the nitrogen assimilation and metabolism leading to a linear correlation that was found between NR activity and $NO_2$ concentration[33]. As reported by Nathalie Durand *et al*.[34], biostimulants had significant effects on nitrate reductase activity even at low concentrations of nitrate. Moshe Halpern *et al*.[35] stated that biostimulants also improve the $NO_3$ assimilation rate by enhancing the enzyme activity. The increase on nitrate reductase activity along with other enzymes was observed on treating barley with biostimulants[36]. Comparable results were also observed in the work of Ertani *et al*.[37], where the genes encoding NR and MDH were up-regulated by the protein hydrolysate in the leaves and their transcript levels accounting for 1.0 to 2-fold increase than in control plants[38]. The biostimulants also alter the microbial community, by supplying micronutrients or other growth factors or by chemically inhibiting specific microbial populations, there by controlling the microbial population. The biostimulants have hindered the breakdown and mineralization of soil organic materials,

resulting in prolonged availability of nitrogen in the soil[39].

## CONCLUSION

In this study, the use of wheat gluten hydrolysate caused substantial increase in shoot and root growth in *Beta vulgaris subs. vulgaris (Linn.)* as compared with the control plants. These results substantiate the increased nutrient absorption by the plant which is reflected in terms of stimulated growth and increase in harvestable yield of the crop plants. Hence, wheat gluten hydrolysate can exhibit a function of a biostimulant and at the concentration of 1mg/L would promote plant growth on application to the soil in the form of a natural fertilizer supplement.

## CONFLICT OF INTEREST

Conflict of interest declared none.

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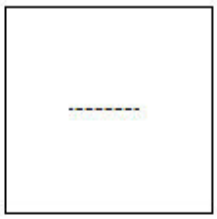


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