S081

Investigation of the positive effects exerted by a new biostimulant on soybean (*Glycine max* L.) seed germination in standard and adverse conditions

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INTRODUCTION

Seed germination that begins with imbibition and ends with root emergence is the first step for plant growth. To complete the germination phase, the proper environmental condition is the most important factor. Germination, growth, root development and emergence uniformity are negatively affected by climate adverse conditions such as extreme temperatures, drought and salinity stress. Temperature is one of the most crucial climatic factors influencing seed germination (Nqobile, 2017). Changes in temperature significantly affect seed germination trough the inhibition of root emergence and post-germination growth in seedlings (Probert, 2000). In order to help seeds to overall stress condition, priming biostimulant treatment has been developed as an indispensable method to preserve the final production.

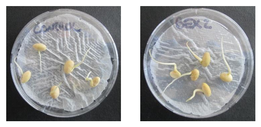

*Fig.1 Seeds untreated and treated with Bex2 (2 ml * kg of seeds^{-1}) incubated for 48 h at 28°C*

AIM

The main aim of this research was to get a better knowledge about priming effect of two biostimulants, Green Has Italia prototypes, on soybean seeds in standard and stress conditions. Soybean (*Glycine max* (L.) Merr) is economically the most important legume crop in the world, providing plant proteins for humans, animals and ingredients for hundreds of chemical products. The biostimulants containing plant-derived amino acids and molybdenum, one based on lignin derivatives, GHI_16_Bex2 (Bex2) and the other one on seaweed extracts, GHI_16_A70V30 (A70V30), were tested in early developing soybean seeds. Germination tests in growth chamber (conducted in standard and stress conditions) and quantitative real time PCR (qPCR) analysis, were conducted to select the most effective product. Since the prototype GHI_16_Bex2 gave the best results in both investigations, RNA-seq analysis on seeds treated with Bex2 on standard and high temperature conditions were conducted, to get new insights in its mechanism of action.

MATERIALS AND METHODS

- ***qPCR***

Seed treatment and germination in Petri dishes (48 h). Five ml of each biostimulant (or water for the control) were added to 12.5 ml of distilled water and then 350 µl of this solution were applied drop by drop to 50 g of soybean seeds, continuously shaken. Then, seeds were placed on Petri dishes with soaked paper (15 ml of distilled water) and incubated for 48 h at 28°C in the dark.

Total RNA isolation and cDNA synthesis. Total RNA from treated and untreated seeds and from corresponding roots (48 h after imbibition) was isolated using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies). cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was used as template in qPCR analyses (Fig. 2).

- ***RNA-seq***

The RNA used for RNA-seq analysis was isolated 24 h after seed imbibition by using TRIzol Reagent® (Thermo Fisher Scientific), with some modifications aimed to improve yield and quality. The seeds used were germinated in Petri dishes under two different conditions: 28°C and 35°C in the dark. Total RNA quality and quantity was checked by using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies) and the quantification of RNA was also confirmed spectrophotometrically. For RNA-seq library preparation 3 µg RNA was used as input material. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated. For data analysis, the index of the reference genome was built using Bowtie v2.2.3 and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12. HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. Then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis of two conditions/groups (three biological replicates per condition) was performed using the DESeq R package (1.18.0) (Fig.3).

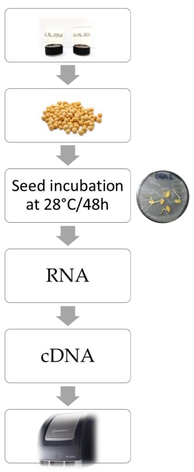

Fig.2: representative diagram of the method employed for qPCR analyses

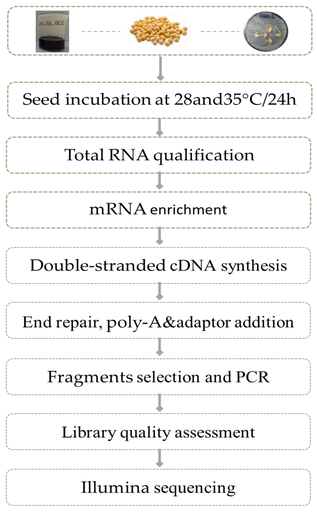

Fig.3: representative diagram of the method employed for RNA-seq analyses

RESULTS AND DISCUSSION

- ***qPCR***

Real time PCR (q-PCR) analysis showed differences in treated seeds/roots compared to untreated ones at gene expression level. The analyzed genes, identified in literature and mainly involved in soybean germination process, are resumed in table 1. In particular Lea protein are low-molecular weight proteins that protect other proteins from abiotic stresses. To evaluate gene expression level during the germination process, seeds and roots were analyzed separately.

The two biostimulants positively modulated some analyzed genes. GHI_16_Bex2 was more effective in particular on radicle in which positively expressed all analyzed genes: PM26 (fold change 11,97±0,255), PM30 (fold change 10,27±0,493), GST (fold change 75,68 ±7,09), PHY (fold change 55,18±9,66) e MAT (fold change 14,47±2,012) in comparison with control. On seeds GHI-16-BEX2 promoted the up-regulation of PM26 (fold change 4,44±0,147), PM30 (2,35±1,297) e GST (fold change 1,93±0,162), but it did not influence the modulation of PHY and MAT.

Tab 1: genes analyzed by qPCR, identified in literature and mainly involved in soybean germination process

Glutathione S-transferase GST 24	GmGST	Abiotic stress response
Seed maturation protein PM30	GmPM30	LEA protein. Abiotic stress response
Maturation-associated protein	GmMAT	LEA protein. Abiotic stress response
Seed maturation protein PM26	GmPM26	LEA protein. Abiotic stress response
Purple acid phosphatase 26-like	GmPHY	Conversion of phytic acid in phosphate available for seed

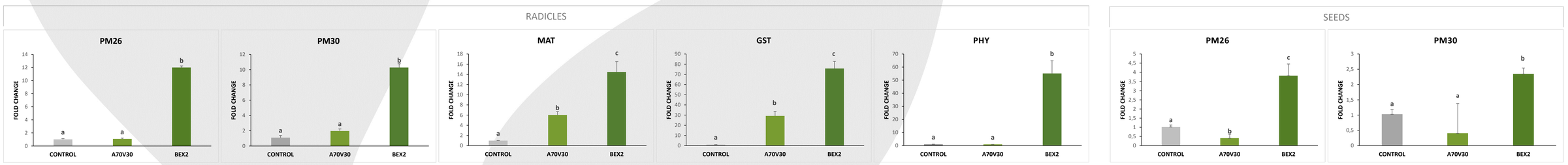

Fig.4: Relative expression of genes involved in germination process in control and in Bex2 and A70V30 treated soybean seeds and roots. Metric bars indicate SE. In each graph, different letters indicate significant differences (t-test; p<0,05)

- ***RNA-seq***

To study the biostimulant action at molecular level, RNA-seq analyses were conducted on both untreated and Bex2-treated seeds incubated 24 hours at different temperatures (28°C and 35°C). Preliminary data analyses showed that Bex2 was able to modulate gene expression at both temperature conditions, however its application was more effective at 35°C as shown in Fig. 4 and 5. In this condition, Bex2 significantly modulated 1100 genes (65 up–regulated and 1035 down-regulated). Among the genes up-regulated by Bex2 treatment, we observed a significant number of those involved in transcription process and RNA biogenesis (>40%) and in stress responses (>20%). On the contrary, most part of the down-regulated genes code for uncharacterized protein (>50%) and photosynthesis (>25%).

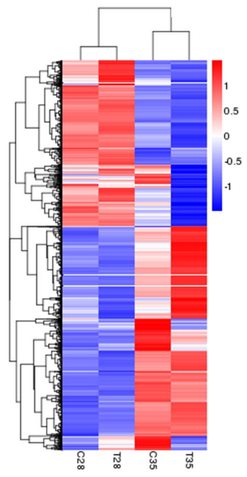

Fig 4: Cluster analysis. The overall results of FPKM cluster analysis, clustered using the log_{10}(FPKM+1) value. Red color indicates genes with high expression levels and blue color indicates genes with low expression levels

DIFFERENTIALLY EXPRESSED GENES (p<0,01)	ALL	UP	DOWN
C35vsC28	2170	1188	982
T28vsC28	82	65	17
T35vsC35	1100	65	1035
T35vsC28	3829	2510	1319

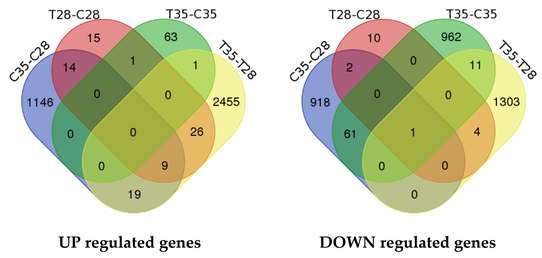

Fig.5: Venn diagrams of commonly and differentially expressed soybean genes upon Bex2 application

CONCLUSIONS

GHI_16_Bex2 is a biostimulant able to modulate the gene expression in early developing soybean seeds, both in standard and in adverse conditions (higher temperature), as demonstrated by qPCR and RNA-seq analyses. Our findings are of paramount importance for the application of Bex2 as a priming biostimulant able to increase seed tolerance to different abiotic stresses typical of extreme conditions. Further agronomic trials are ongoing to evaluate the efficacy of Bex2 in open-field in different climate conditions and soils. All the studies carried out, will provide detailed information about the mode of action and the efficacy of Bex2 from research to the field.

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