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Enhanced lignin and quinone accumulation under varying degree of drought stress influenced by chitosan

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Abstract
Application of 0.25% chitosan used as seed priming in (*Phaseolus vulgaris* L.) grown under varying degree of drought stress enhanced lignin and quinone content in the plant system. Seed primed plants showed positive response to bio primer and enhanced the quinone content in higher concentration compared to control and unprimed. Seed primed sample had lignin in a range of 0.049 to 0.823 and 0.074 to 0.954 mg/g DW in VL Rajma 63 and BR 104 verities respectively. Quinone was found in a range of 0.531 to 1.426 and 0.670 to 1.759 mg/g DW in VL Rajma 63 and BR 104 respectively. These lignin and quinone were secondary metabolites interconnected to strengthen the plant under stress. These lignin and quinone were the phenolic compounds biosynthesized from the phenylpropanoid pathway.

Keywords: BioPrimer, chitosan, phenylpropanoid pathway, secondary metabolites

Introduction
Common bean (*Phaseolus vulgaris* L.) is the important legume crop grown throughout the world as a main dietary source of proteins and amino acids, carbohydrate, dietary fiber, and minerals and help to reduce the risks of serious human diseases (Reyes-Bastidas *et al.*, 2010) [6]. More than 60% of the world's common bean is cultivated under non-irrigated conditions and among grain legumes; common beans are relatively sensitive to drought stress. Drought stress affects the several stages of development either during early growth, during the vegetative development or at flowering or pod/seed filling (flower and pod abortion and yield reduction) and drought is estimated to cause up to 80% yield losses in many regions of the world (Kazai *et al.*, 2019) [4]. Quinones are dietary plant components and electrophilic compound derived the metabolism of phenols and other aromatic amino acids (Lennarz and Lane, 2013) [5]. Red kidney bean (*Phaseolus vulgaris* L.) cultivation is gaining popularity among growers in the Northern Plains and North Western Himalayas of India due to its superior health benefits, and better value-added export opportunities. It is popularly known as "Rajma" and generally grown as a major *Kharif* pulse as well as in spring season. Plants adaptive response to abiotic stress, including drought stress, in part involves stimulation of the biosynthesis of secondary metabolites and induction of endogenous antioxidant enzyme responses (Akula and Ravishankar, 2011) [1]. Chitosan a structural polysaccharide is used as natural stimulants for countering drought stress. Chitosan is natural polymers composed of deacetylated and acetylated unit linked by β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. It is a natural, biodegradable, inexpensive and environmentally friendly with various applications in agriculture by modulating many genes related to plant defense signaling pathways. Shikimate and phenylpropanoid pathway is involved in lignin biosynthesis which gets deposited in the secondary cell walls of all vascular plants. So by analyzing the content of lignin it can be revealed the importance of these two pathways. And the activation of this lignin is depending on the activity of peroxidase and polyphenol oxidase and these enzymes interact with ROS for generation of signal in related to lignification (Frei, 2013) [2]. Lignin and lignin related compound is involved in generation of Quinone in plant (Wozniak *et al.*, 1989) [7]

Keeping in view the above mentioned facts, the present study aims to evaluate the enhancement of lignin and quinone accumulation under varying degree of drought stress influenced by Chitosan. A study shall be also undertaken to evaluate the effects of drought stress, priming agent, and genotypes on the regulation of drought stress response. Further, a study to evaluate the effects of a seed priming application to enhance lignin and will also be

carried out to determine the potential evidence that stimulation of the phenylpropanoid pathway can simultaneously improve abiotic stress resilience as well as health relevant bioactive profiles in red kidney beans.

Method and Material

The present study on **"**Enhanced Lignin and Quinone accumulation under varying degree of drought stress influenced by Chitosan**"** was carried out in controlled conditions in Division of Biochemistry at Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha.

Germplasm collection and experimental setup

The variety VL-rajma 63 was procured from ICAR-VPKVK Almora, Uttarakhand, and BR-104 was procured from the Regional Horticultural Research SubStation, Bhaderwah, SKUAST Jammu. Jammu and Kashmir, India was. The VL-63 variety is grown in the hilly regions as well as central zone of India and gives higher yields. Another variety is recently released by SKUAST-Jammu which is grown in Bhaderwah. The collected seed samples were cleaned for debris and contaminants, air dried and kept in tight container for further studies. VL-Rajma 63 and BR-104 were two genotypes selected for further study under pot conditions. These two germplasm were sown according to treatment in poly grow bags (15*15*30 cm), filled with a mixture of soil, sand, and vermicompost in 2:1:1 proportion. After germination, one healthy seedling was maintained per poly grow bag for each treatment and replications

Irrigation regimes and treatments during common bean cultivation

The Red kidney bean (*Phaseolus vulgaris* L.) cv. VL- Rajma 63 and BR-104 was grown under three form of restricted irrigation (50, 30 and 20% irrigation) for primed and unprimed. 0.25% chitosan seed treatments during the growing cycle in polythene grow bags. For priming the seeds 0.25% chitosan was prepared by dissolving the chitosan in 0.5% of acetic acid and maintained the pH (6-7) by adding required amount of alkali (NaOH, 1M) dropwise. Then the seeds were dipped in this priming solution for 2 hours and kept in orbital shaker for continuous shaking. In this study 7 different treatments were followed including Control were unprimed 50% irrigated, seed primed 50% irrigated, unprimed 30% irrigated, seed primed 30% irrigated, unprimed 20% irrigated and seed primed 20% irrigated

Estimation of lignin content in leaves of chitosan seed primed plant

Lignin content was determined by the method described by Stafford, (1960). 250 mg of dried leaf samples were homogenized with 5 ml of hexane. Homogenates were centrifuged at 10,000 rpm for 15 min and the extraction was repeated thrice for complete removal of chlorophyll pigments and fats contents. Supernatant was decanted and the residues were air dried. 2ml of NaOH was added to the residue and re-extracted at 70-80 °C for 16 h. After cooling 0.45 ml of 2N HCL was added and the pH 7 was adjusted with NaOH. Final volume of 3 ml was made by adding double distilled water and centrifuged at 5000 rpm for 5 min. The supernatant was collected as source of extract. To 0.8 ml of extract 0.8 ml of 0.1 M sodium phosphate buffer pH 7.0 was added. Similarly in another aliquot of 0.8 ml extract 0.8 ml of 0.1N NaOH pH 12.3 was added. The absorbance was measured at 245 and 350 nm the lignin content was calculated by calculating difference between absorbance at 245 and 350 nm at pH 7 and 12.3. Standard curve was prepared by using alkali lignin (100 µg/ml)

Estimation of Quinones content in leaves of chitosan seed primed plantlets

100 mg dried leaf samples were ground to a fine powder with 2 ml of chilled sodium phosphate
buffer (0.1 M, pH 6.6) using pre-chilled mortar and pestle. The supernatant (enzyme extract) was collected by centrifugation at 3000 rpm for 30 minutes at 4 °C. 3 ml of sodium phosphate
buffer (0.1 M, pH 6.6), 3 ml of standard catechol (5 X 10^{-3}M in water) and 1.5 ml of enzyme extract was pipette out in a test tube. It was shaken gently and incubated in water bath for 15 min at 65 °C. 2 ml of samples was withdrawn in duplicate at different time intervals. 4 ml of 0.5 M TCA (Trichloroacetic acid) was added in 60% ethanol (without ascorbic acid) to one and to another corresponding sample added 4 ml of 0.5 M TCA (Trichloroacetic acid) in 60% ethanol (with 0.05 M ascorbic acid). Precipitate was filtered and the absorbance was measured at 400 nm against a reagent blank lacking only extract. Standard curve was prepared using working standard of catechol (1mg/ml)

Result and Discussion

Lignin content in leaves of chitosan seed primed plant

When lignin content in leaf samples of VL Rajma 63 and BR 104 were analyzed (table No.1), it was observed that there was a considerable difference in lignin contents of both varieties when 30th day and 70th day were compared. All the treatments were found to be statistically significantly at ($p<$ 0.05). In VL Rajma 63, least lignin contents were observed in unprimed VL Rajma 63 plant with 50% irrigated, followed by VL Rajma 63 control plant and unprimed VL Rajma 63 plant with 30% irrigated at 0.049 ± 0.002 mg/g DW, 0.115 ± 0.003 mg/g DW and 0.165 ± 0.004 mg/g DW respectively at 30 days of sowing. Whereas highest lignin content was found at 70 days after sowing with a whooping content of 0.823 ± 0.002 mg/g DW in treatment unprimed VL Rajma 63 plant with 20% irrigated followed by 0.822 ± 0.002 mg/g DW in seed primed VL Rajma 63 plant with 20% irrigated. Maximum value recorded at unprimed VL Rajma 63 plant with 20% irrigated which was 7.156 folds more than lignin recorded at VL Rajma 63 control plant. All the treatments of VL Rajma 63 (Control, unprimed 50% irrigated, seed primed 50% irrigated, unprimed 30% irrigated, seed primed 30% irrigated, unprimed 20% irrigated and seed primed 20% irrigated) on 70th day showed increased lignin content which was 4.57, 12.88, 3.19, 4.79, 3.90, 4.19 and 4.39 folds higher compared to 30th day of their respective treatments.

In the variety BR 104, highest lignin content was found in the treatment unprimed BR 104 with 20% irrigated at 0.954 ± 0.000 mg/g FW, followed by unprimed BR 104 with 30% irrigated at 0.88 ± 0.001 mg/g DW both after 70 days of sowing. Unprimed BR 104 with 50% irrigated recorded the least lignin content at 0.074 ± 0.002 mg/g DW followed by 0.096 ± 0.003mg/g DW in BR 104 control plant, both at 30 days after sowing. Maximum value recorded at unprimed BR 104 with 20% irrigated was 12.891 folds more than lignin recorded at BR 104 control plant. All the treatments of BR 104 (control, unprimed 50% irrigated, seed primed 50%

irrigated, unprimed 30% irrigated, seed primed 30% irrigated, unprimed 20% irrigated and seed primed 20% irrigated) on 70th day had increased lignin content of 5.55, 8.64, 3.78, 5.98, 4.36, 5.33 and 4.63 folds compared to 30th day of their respective treatments. Current study showed the severe stress increases lignin content in control range from 0.096 to 0.535 mg/g DW in VL Rajma 63 and In BR 104 it was observed 0.115 to 0.525 mg/g DW. In Seed primed it was found in a range of 0.049 to 0.823 and 0.074 to 0.954 mg/g DW in VL Rajma 63 and BR 104 respectively. And in foliage spray it was observed in a range of 0.076 to 0.791 and 0.105 to 0.761 mg/g DW in both VL Rajma 63 and BR 104 respectively. Moreover, abiotic stress affect the lignification of crops, Peterson *et al*., 1992 reported that increase in lignin under stress in forage legumes. And there is no literature reported to the best of knowledge related lignification in leaves of common bean. In this study seed priming can considered as suitable priming for increasing lignin compared to foliage spray. Lignification facilitates water flow and maintains structural integrity of the xylem vessels during salt stress. So based on role of lignin considered that phenylpropanoid here also play a major role in synthesis of lignin under stress to strengthen the plants and mitigate from the effect of drought stress. This can be further proved by studying the different genes involved in pathway.

Table 1: Lignin content in the seed primed plant leaf sample

Treatment	30 DAS	40 DAS	50 DAS	60 DAS	70 DAS
A	0.115 ± 0.003^{A}	0.208 ± 0.001^{w}	0.219 ± 0.004^{v}	0.254 ± 0.002^{t}	0.525 ± 0.004^{h}
B	0.049 ± 0.002^{B}	0.265 ± 0.000^{s}	0.244 ± 0.005^{u}	0.430 ± 0.002^{n}	0.637 ± 0.003^{e}
C	0.193 ± 0.002^{x}	0.217 ± 0.002^{v}	0.265 ± 0.003^{s}	0.408 ± 0.000^{o}	0.616 ± 0.004^{f}
D	0.165 ± 0.004^{z}	0.487 ± 0.003^{k}	0.345 ± 0.002^{q}	0.466 ± 0.001^{l}	0.790 ± 0.002^{b}
E	0.184 ± 0.001^{y}	0.275 ± 0.001^{r}	0.276 ± 0.003^{r}	0.444 ± 0.002^{m}	0.716 ± 0.003^{c}
F	0.197 ± 0.002^{x}	0.506 ± 0.004^{i}	0.493 ± 0.003^{j}	0.662 ± 0.001^{d}	0.823 ± 0.002^{a}
G	0.187 ± 0.002^{y}	0.383 ± 0.002^{p}	0.386 ± 0.002^{p}	0.574 ± 0.001^{g}	0.822 ± 0.002^{a}
H	0.096 ± 0.003^{F}	0.194 ± 0.002^{z}	0.221 ± 0.002^{x}	0.367 ± 0.003^{s}	0.535 ± 0.002^{l}
I	0.074 ± 0.002^{G}	0.273 ± 0.000^{v}	0.239 ± 0.002^{w}	0.574 ± 0.002^{i}	0.641 ± 0.001^{g}
J	0.186 ± 0.001^{A}	0.211 ± 0.002^{y}	0.243 ± 0.003^{w}	0.374 ± 0.002^{r}	0.703 ± 0.003^{d}
K	0.148 ± 0.002^{E}	0.335 ± 0.004^{u}	0.504 ± 0.002^{n}	0.592 ± 0.002^{h}	0.88 ± 0.001^{b}
L	0.158 ± 0.004^{D}	0.275 ± 0.002^{v}	0.408 ± 0.001^{w}	0.494 ± 0.003^{o}	0.690 ± 0.002^{e}
M	0.179 ± 0.003^{B}	0.550 ± 0.002^{k}	0.569 ± 0.002^{j}	0.681 ± 0.008^{f}	0.954 ± 0.000^{a}
N	0.171 ± 0.002^{C}	0.344 ± 0.004^{t}	0.483 ± 0.003^{p}	0.525 ± 0.002^{m}	0.793 ± 0.001^{c}

*A; Control, B: unprimed 50% irrigated, C: seed primed 50% irrigated, D: unprimed 30% irrigated, E: seed primed 30% irrigated, F: unprimed 20% irrigated and G: seed primed 20% irrigated.

Quinone in leaves of chitosan seed primed plant

The results showed the increased trend of quinone as the stress and days progressed (table No.2) In present study VL Rajma 63 plant showed higher quinone concentration on 70th day in the treatment Seed primed VL Rajma 63 with 20% irrigated (1.426 ± 0.004 mg/g DW) followed by same treatment (1.101 ± 0.004 mg/g DW) on 60th day. Lowest concentration was found in the VL Rajma 63 control plant (0.135 ±0.002 mg/g DW) on 30th day followed by 40th day of VL Rajma 63 control plant (0.170 ± 0.003 mg/g DW). Maximum value recorded at Seed primed VL Rajma 63 with 20% irrigated on 70th day was10.562 folds higher compared to VL Rajma 63 control plant on 30th day. All the treatments found statistically significant at (p< 0.05). All the treatments VL Rajma 63 (Control, unprimed 50% irrigated, seed primed 50% irrigated, unprimed 30% irrigated, seed primed 30% irrigated, unprimed 20% irrigated and seed primed 20% irrigated) on 70th day showed increased quinone content which was 2.02, 1.57, 1.00, 2.19, 1.47, 2.06 and 1.62 folds higher compared to 30th day of their respective treatments.

BR 104 also observed increased concentration of quinone as the stress and days progressed. Chitosan primed samples showed higher concentration of quinone compared to unprimed. In BR 104 variety highest quinone content was observed in the treatment seed primed BR 104 with 20% irrigated on 70th days followed by 60th and 50th day after sowing *i.e.* 1.759 ± 0.003, 1.502 ± 0.002 and 1.442 ± 0.002 mg/g DW respectively. It was found least in the sample BR 104 control plant (0.270 ± 0.003 mg/g DW) on 30th day followed by BR 104 control plant (0.302 ± 0.002 mg/g DW) on 50th day. Maximum value recorded at seed primed BR 104 with 20% irrigated on 70th day was 6.51 folds higher compared to seed primed BR 104 with 20% irrigated on 30th day. All the treatments of BR 104 (control, unprimed 50% irrigated, seed primed 50% irrigated, unprimed 30% irrigated, seed primed 30% irrigated, unprimed 20% irrigated and seed primed 20% irrigated) on 70th day showed increased quinone content which was 2.02, 1.27, 1.39, 1.62, 1.36, 1.50 and 1.89 folds higher compared to 30th day of their respective treatments. Current study showed the drought stress gradually increases quinone content in all treatment including control as day progressed. Control plant had quinone range from 0.135 to 0.273 mg/g DW in VL Rajma 63 and In BR 104 it was observed 0.270 to 0.314 mg/g DW. In Seed primed it was found in a range of 0.531 to 1.426 and 0.670 to 1.759 mg/g DW in VL Rajma 63 and BR 104 respectively. And in foliage spray it was observed in a range of 0.536 to 1.315 and 0.670 to 1.648 mg/g DW in both VL Rajma 63 and BR 104 respectively. Similarly in unprimed it was in a range of 0.271 to 1.092 and 0.404 to 1.203 mg/g DW respectively. In this study observed that the concentration of quinone was increased as the day progressed and observed that the speed primed plant showed higher accumulation of quinone compared to foliage spray and unprimed plants. And in control plant it was lesser concentration it may be due to less stress. Quinone reductases are the flavor protein that protect from the oxidative stress (Heyno and Fluhr, 2013) [3].

Table 2: Quinone content in the seed primed leaf sample

Treatment	30 DAS	40 DAS	50 DAS	60 DAS	70 DAS
A	0.135 ± 0.002^{E}	0.170 ± 0.003^{D}	0.242 ± 0.002^{C}	0.260 ± 0.001^{B}	0.273 ± 0.003^{A}
B	0.271 ± 0.004^{A}	0.336 ± 0.003^{z}	0.375 ± 0.001^{y}	0.400 ± 0.002^{w}	0.424 ± 0.002^{v}
C	0.536 ± 0.003^{r}	0.670 ± 0.003^{o}	0.509 ± 0.003^{t}	0.512 ± 0.002^{st}	0.534 ± 0.004^{r}
D	0.397 ± 0.002^{x}	0.497 ± 0.002^{u}	0.644 ± 0.002^{q}	0.700 ± 0.002^{n}	0.869 ± 0.002^{j}
E	0.664 ± 0.003^{p}	0.835 ± 0.003^{k}	0.776 ± 0.004^{m}	0.823 ± 0.003^{l}	0.975 ± 0.002^{f}
F	0.531 ± 0.002^{s}	0.997 ± 0.003^{e}	0.910 ± 0.002^{h}	0.963 ± 0.001^{g}	1.092 ± 0.001^{c}
G	0.880 ± 0.003^{i}	1.163 ± 0.003^{b}	1.043 ± 0.003^{d}	1.101 ± 0.004^{bc}	1.426 ± 0.004^{a}
H	0.270 ± 0.003^{D}	0.330 ± 0.004^{z}	0.302 ± 0.002^{C}	0.307 ± 0.002^{B}	0.314 ± 0.002^{A}
I	0.404 ± 0.004^{y}	0.663 ± 0.003^{s}	0.508 ± 0.002^{w}	0.510 ± 0.003^{vw}	0.515 ± 0.003^{v}
J	0.670 ± 0.003^{p}	0.831 ± 0.001^{o}	0.905 ± 0.003^{d}	0.909 ± 0.002^{e}	0.934 ± 0.003^{f}
K	0.535 ± 0.004^{p}	0.502 ± 0.002^{o}	0.640 ± 0.003^{i}	0.701 ± 0.002^{h}	0.869 ± 0.004^{g}
L	0.804 ± 0.002^{u}	0.835 ± 0.002^{x}	1.043 ± 0.001^{t}	1.052 ± 0.004^{q}	1.092 ± 0.003^{n}
M	0.803 ± 0.003^{r}	0.834 ± 0.002^{o}	1.303 ± 0.001^{m}	1.285 ± 0.002^{l}	1.203 ± 0.001^{k}
N	0.930 ± 0.003^{k}	1.005 ± 0.005^{j}	1.442 ± 0.002^{c}	1.502 ± 0.002^{b}	1.759 ± 0.003^{a}

*A; Control, B: unprimed 50% irrigated, C: seed primed 50% irrigated, D: unprimed 30% irrigated, E: seed primed 30% irrigated, F: unprimed 20% irrigated and G: seed primed 20% irrigated.

Conclusion

This study demonstrates that the application of 0.25% chitosan as seed priming under restricted irrigation in red kidney bean (*Phaseolus vulgaris.* L) cv. VL Rajma 63 and BR 104, cultivation in a rainout shelter enhanced the accumulation of lignin and quinone. Lignification facilitates water flow and maintains structural integrity of the xylem vessels during drought stress. Based on the study result can consider that the chitosan with 0.25% seed priming increased the accumulation of quinone for mitigation of drought stress and protect the plant cellular damage.

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