Journal of Stress Physiology & Biochemistry, Vol. 19, No. 1, 2023, pp. 141-153 ISSN 1997-0838

ORIGINAL ARTICLE

OPEN ACCESS

Differential hikes in phenolic and flavonoid compounds in germinating soybean (*Glycine max*) seeds under abiotic stresses

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Received October 22, 2022

Background: The current study focuses on the participation of total phenolic content (TPC), total flavonoid content (TFC) as well as antioxidant activity in germinating soybean seeds under abiotic stress.

The context and purpose of the study: For this study, soybean seeds were subjected to three abiotic stresses namely drought, flood, and salinity for 3 days.

Method: The estimation of TPC and TFC was performed by FTIR and spectrophotometric method. The spectrophotometric estimation of TPC and TFC was performed by Folin Ciocalteu and Aluminium Chloride method respectively.

Results: The highest TPC (68.78 µg gallic acid/ml) and TFC content (6.23 µg quercetin/ml) found in drought treated seeds for one day. In terms of the DPPH scavenging assay, the highest (67.63%) and lowest (46.83%) percentage of inhibition was observed on the1st and 3rd day of salinity stress respectively. Results of the current study also showed a strong positive correlation between TPC and TFC analyzed by FTIR and Spectral data.

Conclusion: The study supports the role of phenolic and flavonoid in germinating soybean seeds under abiotic stress.

Key words: Abiotic stress, Phenolic, Flavonoid, FTIR, Soybean (Glycine max).

Soybean (*Glycine max*) is one of the most important cash crops and the third-largest leguminous crop cultivated in India (Khan *et al.*, 2009, Mutava *et al.*, 2015, Waqas *et al.*, 2019). Soybean is one of the major cash crop and it is a good source of oil and proteins for humans, cattle and aquaculture (Deshmukh *et al.*, 2014; Todayoshi and Goldsmith 2009). Abiotic stresses like drought, flood, and salinity hamper the plant growth and lower the yield of soybean worldwide (Waqas *et al.*, 2019).

The demand for food increases with time due to the increase in population. The ecological balance is disturbed by the severe climatic changes in the environment. Such adverse climatic changes impose various kinds of abiotic and biotic stresses on plants. Crop plants are extremely sensitive to these abiotic stresses such as drought, heat, cold, salinity, UV radiation, oxidative stress, mechanical stress, nutrient stress, and phytohormone- mediated stress (Bej and Basak 2014) which affect the plant performance and lead to reduced productivity. These abiotic factors are the major cause of severe losses in agriculture throughout the world (Barnabas *et al.*, 2008; Athar and Ashraf 2009). Adverse changes in the environmental conditions are also reducing the productivity of soybean crop.

Plants imposed by abiotic stresses shows anxious metabolism. Plant metabolomics plays a very important role in resisting adverse effects generated by stress conditions (Austen *et al.*, 2019; Xu *et al.*, 2022). To defeat with such drastic unfavorable environmental conditions plant exhibit alteration at physiological, molecular, and cellular level (Sahitya *et al.*, 2018). The abiotic stress factors including drought, soil flooding, salinity and extreme temperatures, induce the major amendment in the plant metabolome make–up (Zandalinas *et al.*, 2022). For crop development, knowledge of the acute function of primary and secondary metabolites in the stress tolerance process is required. The plant produces secondary metabolites due to distinct physiological alterations (Zandalinas *et al.*, 2018). These secondary metabolites monitor the plant growth, development, survival and protect the plants facing various stressed environments (Athar and Ashraf 2009). The secondary metabolites can be classified into three groups namely phenolic compounds (flavonoids and phenylpropanoids), terpenes (isoprenoids), and nitrogen-containing compounds (cyanogenic glycosides, alkaloids, and glucosinolates). Among these, Phenolic compounds play an important role in plant growth, reproduction, and resistance to abiotic and biotic stress (Achakzai *et al.*, 2009; Gimenez *et al.*, 2014). Metabolomics is one of the important way by which the molecular mechanism of stress resistance can be analyzed adequately (Deshmukh *et al.*, 2014).

In India, monsoon irregularity, groundwater depletion, and increasing population are the main reason for water scarcity and drought stress. Soybean production was reduced worldwide by 33.1-12.2% due to drought (Sahitya *et al.*, 2018). Under drought conditions, free radicals get increase and starts to steal electrons from lipids present in the cell membrane which shows damage and leakage in the plant cell membrane. This process is known as lipid peroxidation. (Gharibi *et al.*, 2016; Sarker and Oba 2018).

The commencement of flood is due to overindulgent irrigation, the low infiltration rate of soil and heavy rainfall. In submerged conditions, the rate of photosynthesis lowers because of the lack of carbon dioxide which further shows leaf chlorosis. The majority of soybean varieties are sensible to flooding and responsible for chlorosis, necrosis, stunting, defoliation, reduced nitrogen fixation, and plant death. Worldwide 16% of soybean production turns down due to flood stress. Soybean seed shows lower seed germination and survival rate as oxygen supply is not properly maintained in flooding conditions (Wu *et al.*, 2017). In addition to this fungus also affect the seed germination and seedling emergence in plants including *Fusarium, Rhizoctonia, Phomopsis, Pythium* and *Phytophthora* (Hussain and Farzana 2019, Wu et al., 2017).

Excess use of fertilizers and obstructive irrigation are the two main reasons behind cropland salinity. Currently near about 800 million hectares of the land in the world are influenced by salinity (Khan *et al.*, 2009). Salinity is responsible for poor plant growth and reduced crop yield. Further, salinity is also associated with oxidative

stress, osmotic stress and toxicity (Neves *et al.,* 2010). During the salinity stress Na^+ and Cl^- ions aggregate in plant tissues. These increased Na^+ concentrations prohibit the uptake of K^+ ions which is crucial for plant growth and development. In these conditions, crop yield is reduced and can lead to plant death. The plant increases the production of reactive oxygen species (ROS) in such stressful situations. These ROS pull down by antioxidant agents like phenolic and flavonoid (Gupta and Huang, 2014, Shah and Smith, 2020, Waqas *et al*., 2019). Salinity stress can lower the 40% of soybean yield (Cheng *et al.*, 2020).

Abiotic stress like drought and salinity in plants shows oxidative damage (Chen *et al.*, 2013; Sarker and Oba, 2018). The oxidative damage further causes the generation of ROS like OH^-, H_2O_2 and O_2. This accumulated ROS can directly attack membrane lipid, deactivate metabolic enzymes and damage nucleic acid, leading to cell death (Chung *et al.*, 2020). The plant encountered in such stressed conditions tries to defeat oxidative damage by the antioxidative defense system. This antioxidative defense of the system lowers the destruction created by ROS (Khan *et al.*, 2009). Secondary metabolite like phenolic and flavonoid helps the plant to cope with the stressed condition by detoxifying the ROS (Swigonska *et al.*, 2014; Sarker and Oba, 2018). Therefore, the present investigation has been carried out to evaluate the role of secondary metabolites in mitigating the adverse effect of various stress conditions like drought, flood, and salinity in soybean. The objective of the present study was to evaluate the level of total phenolic and flavonoid components in germinating soybean under abiotic stress by FTIR and colorimetric analysis. A correlation is shown between total phenolic and flavonoid components analyzed by FTIR and spectral data. The study also focused on the antioxidant activity of total phenolic and flavonoid components in germinating soybean seeds under the abiotic stress condition.

To the best of our knowledge, this is our first report in which FTIR was used to examine the effect of three abiotic stresses, namely drought, flood and salinity on phenolic and flavonoid components in soybean during the germination period. The results of the study could be able to evaluate the role of phenolic and flavonoid component in defeating abiotic stress.

MATERIALS AND METHODS

Plant materials and treatments

Soybean seeds were obtained from the Krishi Seva Kendra, Akola, Maharashtra, India. Healthy and equal-sized seeds were chosen, rinsed twice with sterilized distilled water to remove dust and surface sterilized with 0.1 % $HgCl_2$ for 10 minutes. After that, these seeds were thoroughly rinsed and soaked in sterilized water for six hours. Sterilized seeds were placed in clean sterilized petri plates with two layers of moistened filter paper. Ten seeds were added in each petri dish.

For the drought stress treatment, the soaked soybean seeds were placed in sterilized petriplates with dried filter paper (Hackenberg *et al.*, 2015; Hivrale *et al.*, 2016; Zhou *et al*., 2010). For the flood stress treatment, the seeds were kept in a sterilized container with the water level maintained 5 cm above the seed surface (Pires *et al*., 2018; Tan *et al.*, 2016; Wu *et al.*, 2017) and for the salinity stress, two ml of 200 mM NaCl was sprinkled on filter paper and soaked seeds kept on that. The controlled seed was watered regularly. Along with the control seeds, the stress treatment was continued for 1, 2, and 3 days. Each experiment was repeated three times.

Sample Preparation

Soybean seeds (100 mg) were crushed in 1 ml of 80% methanol for 1 hour, after this seeds were sonicated for 10 minutes and centrifuged at 8000 rpm for 10 minutes. The supernatant was collected and filtered through a 0.45µm syringe filter, and the extracts were stored in the dark at -20℃ for further analysis (John *et al*., 2017; Varela *et al.*, 2016).

Total Phenolic Content

The Folin–Ciocalteu method was used to determine the total phenolic content of the extract (Kamtekar *et al.*, 2014; Baba and Malik, 2015). Methanol was added to 0.5 ml of seed extract to make the volume 1 ml, and 0.5 ml of Folin–Ciocalteu reagent was added and mixed well. 1.5 ml of 20% sodium carbonate was added, and the volume was made up to 10 ml with distilled water before incubating for 1 hour in the dark. After incubation,

absorbance was measured at 750 nm using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer instrument. The measurement was carried out in triplicate. The control was made with a reagent blank and solvent. Gallic acid (50µg/ml) was used as a control. The total phenolic content was calculated as µg of gallic acid equivalent weight (GAE) per ml.

Total Flavonoid Content

The aluminum chloride colorimetric assay was used to determine the total flavonoid content (Kamtekar *et al.*, 2014; Baba and Malik, 2015; Abdel-Moemin, 2016). In 0.5 ml of seed extract, Methanol was added to make the volume 1 ml, and 0.3 ml of 5% sodium nitrite was mixed and incubated for 5 minutes before adding 0.3 ml of 10% aluminum chloride and left stable for 6 minutes. Finally, 2 ml of 1 M sodium hydroxide was added, and the volume was increased to 10 ml with distilled water. The absorbance was measured at 510 nm using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer. The blank was created with distilled water. As a control, 25 µg/ml of quercetin was used.

FT-IR analysis and spectral collection

The FTIR spectra of extract of germinating soybean seeds were recorded in an FTIR instrument (Model/Make: Bruker, Germany), with PC-based software controlling instrument operation and data processing. Using KBr, a small amount of powdered seed samples were formed into pellets for FTIR analysis, and a thin film was formed by applying pressure. Infrared transmittance data was collected over a wave number range of 4000 cm^{-1} to 400 cm^{-1}. All samples were examined in triplicate, with plain KBr pellets serving as a control. To identify the functional groups present in the sample, the spectral data were compared to a reference (Santos *et al.*, 2019).

DPPH free radical scavenging activity

The stock solution was made by dissolving 22 mg of DPPH in 50 ml of methanol and keeping it at – 20°C until needed. In methanol, different concentrations (0.1, 0.2 ml ----- to 1 ml) of extracts were prepared. Each extract was combined with 3.9, 3.8 ----- 3 ml DPPH respectively. This mixture was allowed to react for 30 minutes before being measured at 515 nm. In the control sample, the extract was not used. The following formula was used to calculate scavenging activity (Ahmed *et al.*, 2012).

DPPH radical scavenging activity (%) = [(Abs control – Abs sample) / Abs control] x 100

$Abs_{control}$=The absorbance of DPPH radical + methanol

Abs_{sample} = The absorbance of DPPH radical + extracts

Statistical Analysis

Every sample was collected and examined in triplicate. Pearson's correlation test was used to investigate the relationship between total phenolic and flavonoid content. The mean standard deviation was used to analyse the results.

RESULTS

Total Phenolic Content

Results presented in figure 2 revealed an analysis of the total phenolic content (TPC) of methanolic extract of germinated soybean seeds under drought, flood, salinity and control (without stress) conditions. The Gallic acid standard graph is shown in figure 1. The total phenolic content of all stress-treated and control seeds varied according to the treatments. On the first day, the total phenolic content was 13.68 µg/ml in the control while it was observed 68.78µg/ml, 6.25µg/ml, and 47.09µg/ml for drought, flood and salinity respectively. All stress-treated soybean seeds had upregulation in phenolic content except flood when compared to control seeds. On the second day, the observed total phenolic content values were 14.85µg/ml, 24.33µg/ml, 3.03µg/ml, and 7.72µg/ml for control, drought, flood and salinity respectively. As compared to the control set higher TPC was observed under stress conditions and showed significant changes in phenolic content on the second day of germination. Similarly, on the third day of germination, there were significant differences observed in TPC content between the stress-treated seeds and the control set.

Total Flavonoid Content

Figure 4 shows the results of the total flavonoid content of methanolic extract of germinated soybean

seeds under drought, flood, salinity and control conditions. In terms of total flavonoid content, we found significant differences between all stress-treated and control soybean seed. The standard graph of quercetin is shown in figure 3. The total flavonoid content in the control sample was found to be 1.55µg/ml, 6.23µg/ml in drought,1.07µg/ml in flood and 4.28µg/ml in salinity on the first day. On the second day, total flavonoid content was determined to be 1.46µg/ml in the control, 2.92µg/ml in the drought, 0.68µg/ml in the flood and 1.43µg/ml in the salinity. Total flavonoid content increased by twofold in the drought stress compared to the control seeds, dropped dramatically in flood and was nearly identical to 1.43µg/ml for salinity. The total flavonoid content observed in the control condition on the third day of stress treatment to germinating soybean seeds was 2.42µg/ml. We found differences in TFC content of all stress-treated seeds compared to the control condition. The highest total flavonoid content was 2.80µg/ml in drought, 1.70µg/ml in flood and 1.73µg/ml in salinity. The drought shown the highest total flavonoid content on the last day of stress treatment. Salinity causes a very minor increase in total flavonoid content when compared to flood (1.70µg/ml). The differences in total flavonoid content between salinity and flood are negligible. Total flavonoid content increased in flood stress compared to total phenolic content, indicating its role in flood stress.

DPPH Free Radical Scavenging Activity

In our investigation, free radical scavenging activity of methanolic extract of germinated soybean seeds under drought, flood, salinity, and control conditions varied very slightly 46.63-67.63%. The highest activity was found on the 1st day (67.63%) and it was lowest on the 3rd day of the salinity stress. The graph of DPPH free radicle scavenging activity is shown in figure 5.

FT-IR

The functional groups of the active components present in the extract was analysed by using the physicochemical technique FT-IR (Fourier transform infrared) spectroscopy. The functional groups of the components were separated based on the ratio of its peak while the extract was passed through FT-IR. The presence of N-H, O-H, C=C, C-H, C-O, and CH_3 functional groups were identified and confirmed by FT-IR spectral study. The FTIR spectra of germinating soybean seeds under control and stress conditions on 1st, 2nd and 3rd day are shown in figures 6A, 6B, and 6C respectively. The peak values of obtained functional groups compared with standards is shown in table 1.

Correlation

The absorbance values obtained from colorimetric and FTIR data are tabulated in table 2. The correlation matrix for phenolics and flavonoids by colorimetric and FTIR data is shown in Table 3. A strong positive correlation was observed between the FTIR and spectral data analysed for content of total phenolic and flavonoid components. Also drought and salinity values showed an excellent correlation between FTIR and colometric data.

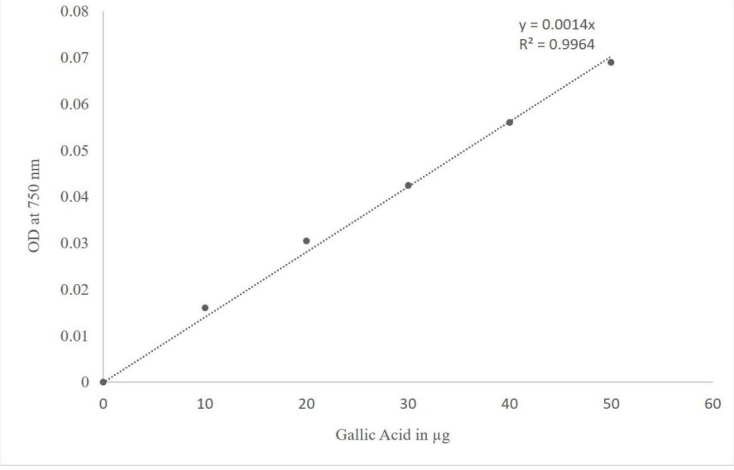

Figure 1 Standard curve of Gallic Acid

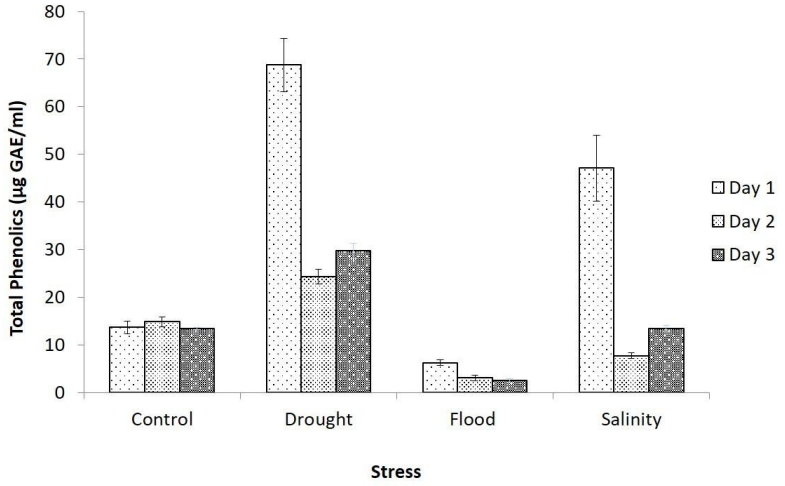

Figure 2 Total phenolic content of germinating soybean seed under abiotic stress condition with the control sample. (GAE- Gallic acid equivalent)

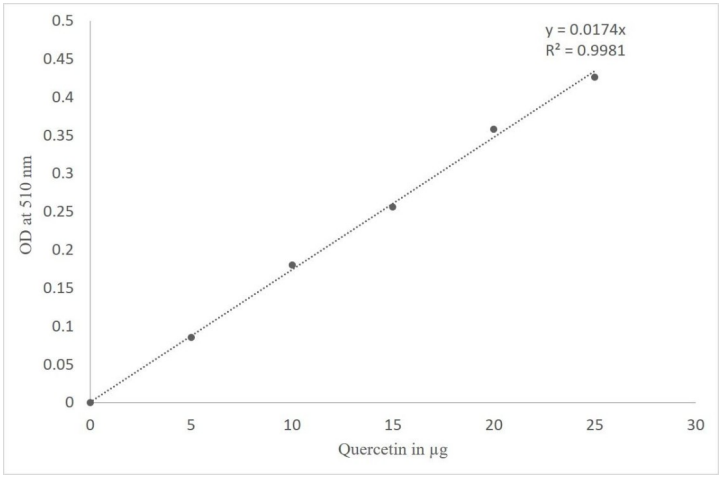

Figure 3 Standard curve of Quercetin

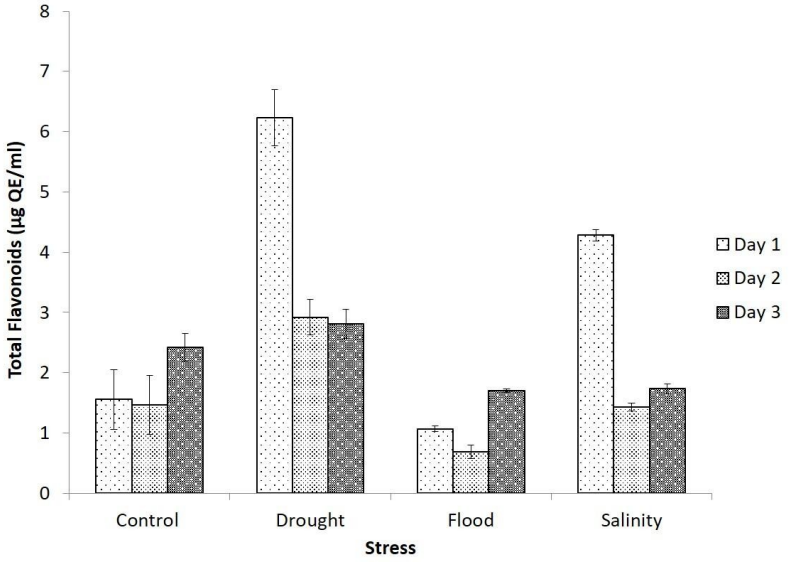

Figure 4 Total flavonoid content of germinating soybean seed under abiotic stress condition with the control sample. (QE- Quercetin equivalent)

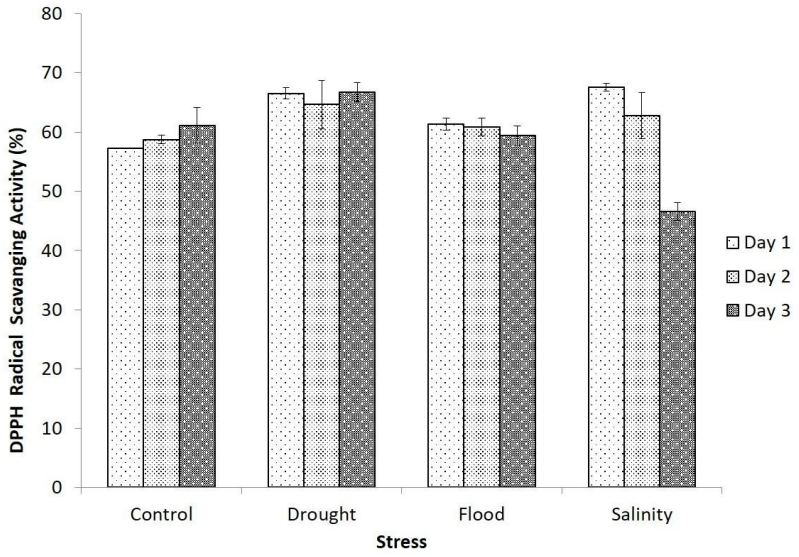

Figure 5 DPPH Free Radical Scavenging Activity

Table 1. FTIR peak assignment table of all stress-treated geminated seeds compared with standard chart

Functional groups	C_1	D_1	F_1	S_1	C_2	D_2	F_2	S_2	C_3	D_3	F_3	S_3
Unknown	3644.44	---	3618.63	---	3678.53	3732.25	3618.86	3666.85	3715.08	3735.31	3734.10	3734.70
Free alcohol –OH stretching	3418.05	3385.44	3425.75	3386.16	3421.45	3384.77	3427.79	3420.21	3410.25	3384.54	3419.49	3416.13
Intramolecular bonded alcohol –OH stretching		2925.87	2923.91	2924.82	2924.51	2924.89	2922.70	2924.69	2924.73	2924.79	2924.02	2924.33
C-H stretching alkane	2853.28	2855.47	2852.87	2853.86	2853.92	2854.03	2850.59	2852.94	2853.95	2853.76	2853.18	2853.13
C≡C, C≡N stretching	2256.30	2233.02	2256.16	----	2231.77	2231.86	2258.32	2257.11	2232.25	2232.64	2233.01	2256.26
C=N stretching imine/oxime or C=O stretching conjugated ketone or alkenes	1613.40	1618.82	1616.87	1608.77	1616.82	1616.73	1618.17	1615.84	1619.82	1618.18	1610.89	1619.59
Alcohols C-O stretch	1047.83	1053.34	1050.98	1053.20	1053.11	1052.20	1028.80	1052.50	1053.66	1051.98	1071.73	1053.17

Table 2. FT-IR and colorimetric data of the germinating soybean seeds under drought, flood, salinity stress with controlled condition.

Sample	FT-IR data		Colorimetric data	
	Phenolics	Flavonoids	TPC	TFC
C1	0.0105	0.0145	0.0109 ± 0.0009	0.0176 ± 0.003
C2	0.0127	0.0168	0.0117 ± 0.0007	0.0163 ± 0.0002
C3	0.0105	0.0213	0.0107 ± 0.0033	0.022 ± 0.002
D1	0.0447	0.0555	0.0485 ± 0.0038	0.055 ± 0.004
D2	0.0172	0.0259	0.0181 ± 0.0010	0.0263 ± 0.0025
D3	0.0218	0.0273	0.0218 ± 0.0010	0.0253 ± 0.0020
F1	0.0061	0.0105	0.0058 ± 0.0004	0.0103 ± 0.0004
F2	0.0043	0.0078	0.0036 ± 0.0003	0.007 ± 0.001
F3	0.0034	0.0168	0.0032 ± 0.0003	0.0158 ± 0.0002
S1	0.0366	0.0404	0.0337 ± 0.0046	0.0381 ± 0.0008
S2	0.0083	0.015	0.0068 ± 0.0004	0.0134 ± 0.0005
S3	0.0105	0.0177	0.0107 ± 0.0004	0.0160 ± 0.0007

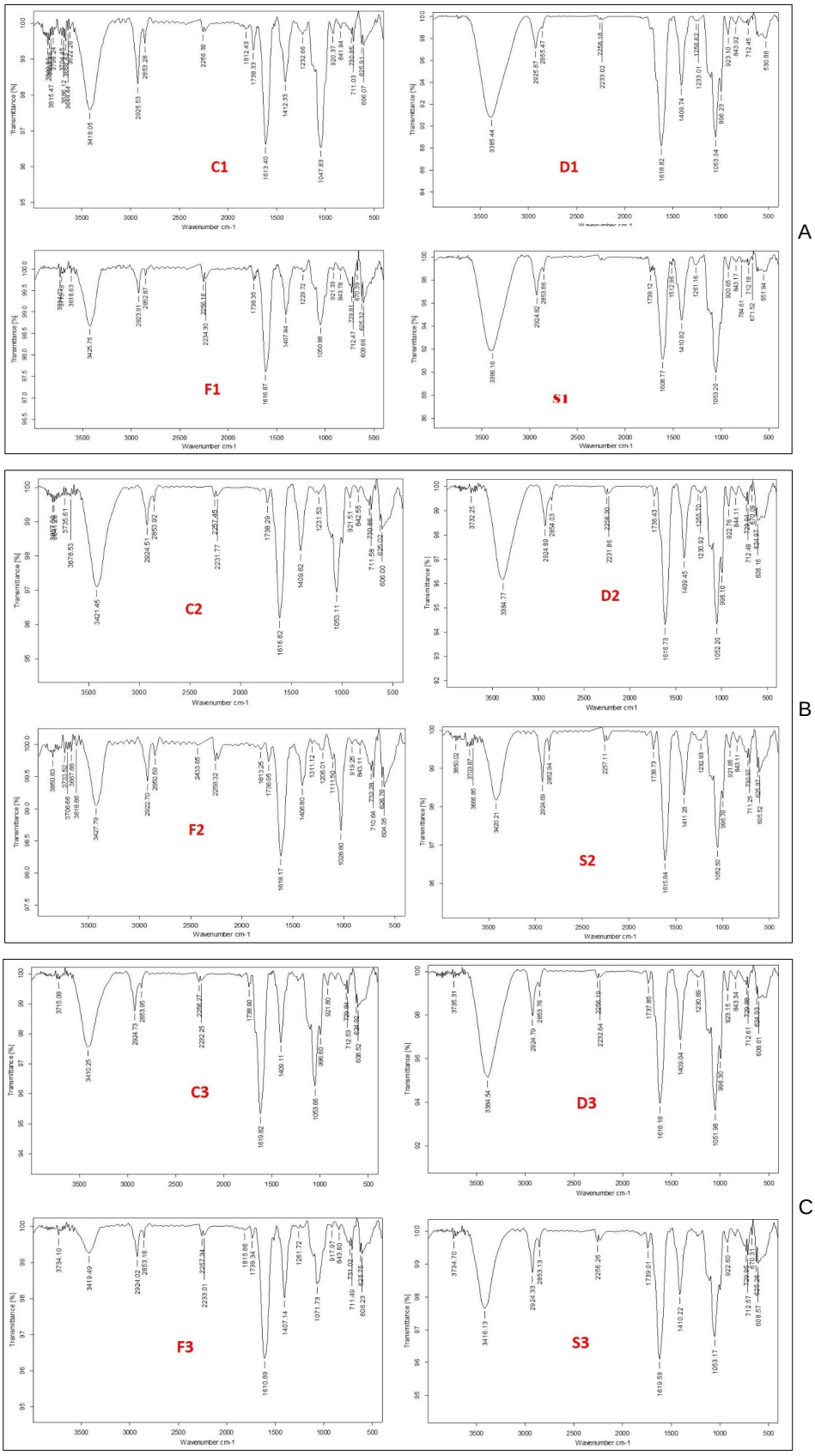

Figure 6 A, B, C Fourier-Transform Infrared Spectroscopy analysis of the germinating soybean seeds treated with abiotic stress along with the control extract of the sample (C-control, D-drought, F-flood and S-salinity)

Table 3. Pearson's linear correlation coefficient (R) matrix analysis between FT-IR and colorimetric data for total phenolic and flavonoid components of germinating soybean seed under abiotic stress.

Variables		FTIR		Colorimetric data	
		Phenolics	Flavonoids	Phenolics	Flavonoids
FTIR	Phenolics	1			
	Flavonoids	0.969433049	1		
Colorimetric data	Phenolics	0.993844241	0.977827801	1	
	Flavonoids	0.961616806	0.994163144	0.976050407	1

DISCUSSION

In the life of a plant, germination is a crucial stage. A developing seedling must contend with environmental stress, microbial attack, and nutrient depletion. The developing seedling defends itself against biotic and abiotic stress factors by synthesizing phenolic compounds via multiple metabolic pathways in such a hostile environment. Our research reveals the role of phenolic and flavonoid content in abiotic stress in germinating soybean seeds. Under drought, flood, and salinity stress, total phenolic and flavonoid component as well as antioxidant activity of sprouting soybean seeds were assessed for 1, 2, and 3 days. The phenolic and flavonoid content increased during drought and salinity stress, implicating a function in maintaining osmotic potential and avoiding oxidative damage, according to our findings.

The principle of this spectroscopy is based on the vibrations of a molecule energized by infrared radiation at a specific wavelength range (Santos *et al.*, 2019). It was based on the peak values in the region of IR radiation. FTIR spectroscopy is confirmed to be the firm and precise method for the detection of bioactive compounds (Muthukumaran *et al.*, 2017)

According to the findings of this study, the phenolic and flavonoid content of the sprouting soybean seed under drought stress was maximum on the first day. Gharibi *et al.* (2016) looked into the high positive link between TPC and TFC in drought-stressed Achillea species. Under severe drought stress, *A. millefolium* had the highest DPPH activity. Genotype KCa-5 exhibited the highest TPC and the largest percentage of DPPH radical scavenging activity in chilli seedlings during drought stress, according to Sahitya *et al.* (2018), whereas genotype KCa-1 had the lowest TPC and DPPH activity. There was also a strong positive correlation between DPPH activity and total phenolics among these genotypes. TPC, TFC, and DPPH levels in *A. tricolour* leaves increased considerably with the intensity of drought stress according to Sarker and Oba (2018). TPC and TFC were studied in the fall season of two native shrubs, *L. divaricata and L. chilense,* by Varela *et al.* (2016). TPC was higher *in L. divaricata* leaves than in *L. chilense* leaves only in autumn, and TFC was higher in *L. divaricata* leaves than in *L. chilense* leaves only in autumn, according to the study. These references are relevant to our findings.

In contrast to ZH13, Chen *et al*. (2013) reported high total phenolic levels in BB52 soybean leaves during salt stress. Mild salt stress enhanced the phenolic content and antioxidant activity of *S. mirzayanii* leaves, according to Valifard *et al.* (2014) whereas excessive salinity ($9.1 dsm^{-1}$) lowered both of these parameters. Neves *et al.* (2010) discovered that raising the concentration of NaCl in soybean roots from 100 to 200 mM enhanced TPC by roughly 50%. Flavonoid production rose when salinity levels increased in *Carthamus tinctorius* leaves, according to Shaki *et al*. (2018). Salinity stress increased total phenolic and flavonoid content in germinating soybean seed on the first day in our study, however the increase was less than drought stress.

Increased TPC and TFC demonstrated that they are important in preventing ROS damage to cellular structures during dryness. Varela (2016) found the similar results. In the case of salinity, TPC and TFC showed a positive association, which explained their involvement in the plant-salinity interaction. Because of the hydroxyl group present in their structure, TPC and TFC accumulate to prevent oxidative damage and act as a free radical scavenger implying their role in maintaining osmotic potential and avoiding oxidative damage. Chen *et al.*, (2013), Valifard *et al.* (2014), Neves *et al.* (2010), and Shaki et al. (2018) all came to similar conclusions. The positive association of TPC and TFC in the case of drought and salinity, according to Gharibi *et al.* (2016) may be attributed to the sharing of a same biosynthetic route.

During flood stress, the flavonoid level increased marginally on the third day, but the phenolic content increased dramatically. For the overall phenolic and flavonoid components, there was a good correlation between the FTIR and Spectral data. During flooding and drought, the polyphenol content and superoxide anion scavenging percent of sweet potato leaves decreased, according to Lin *et al.* (2006). Flooding caused a significant increase in TPC and TFC in strong submerged tolerant rice varieties, according to Tan *et al.* (2016). Under submergence, Banerjee *et al.* (2015) studied the upregulation of phenolic and flavonoid components in rice. According to Lim *et al.* (2017) TPC of mature leaves in *Macaranga pruinosa*was substantially greater during the rainy season.

Our research's main goal is to offer a spoonful of information toward the development of a new stress-resistant soybean variety that can endure abiotic stress while also enhancing yield.

CONCLUSION

FTIR could be a powerful tool for analyzing secondary metabolites expressions in abiotic stress conditions. According to our findings, phenolics and flavonoids significantly increased under abiotic stress hence exhibiting their role in defense against environmental adverse.

ACKNOWLEDGMENT

We would like to thank the Dr. Babasaheb Ambedkar Research and Training Institute (BARTI) in Pune for providing financial support through the Babasaheb Ambedkar National Research Fellowship (BANRF). We'd also like to thank the Indian Institute of Technology, Bombay's (IITB) Sophisticated Analytical Instrument Facility (SAIF) for letting us use their FTIR facility.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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