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Effect of induced stress treatments on *Zingiber officinale* Rosc. cv-Varada with respect to its growth dynamics and phytochemical characterization

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Extensively speaking, environmental stresses have a significant negative impact on agriculture. Plants have developed a multitude of defense responses that allow them to adapt, survive and reproduce under stress conditions. In the present study the effect of different individual stress treatments and their combined treatments on the yield, growth antioxidant activity and phytochemicals of (*Zingiber officinale* cv-varada) were analyzed. A greenhouse experiment was conducted with 12 replications of each trial. The different stress treatments were attempted using Salicylic acid and Zinc Sulphate (10^{-2} & 10^{-3} Molar). Along with these two, drought is also taken as a third stress. The results of Individual and combination of stress treatments were obtained. The present study gives an idea about the effect of individual as well as combined stress treatments in both morphological (yield and growth parameters) antioxidant activity and the phytochemicals. A difference in the total phenolics, total flavonoids and antioxidant activity was observed in different trials. This also gives a clear picture about cross tolerance in which one stress influenced the effect of another.

Key words: Antioxidant, Cross tolerance, Drought, Ginger, Phytochemicals

Plants are a potential source of natural antioxidants (Ghasemzadeh, 2010). Spices contain phytochemicals commonly known as antioxidants such as polyphenols, phenolic acids, tannins, flavonols, isoflavones and curcuminoids . The high proportion of these antioxidants is the reason of various health benefits imparted by the spices. The antioxidants present in spices are capable of neutralizing the free radicals by donating required number of electrons to stabilize them (Shirazi *et al.,* 2014).

Phytochemicals are bio- active chemicals of plant origin. They are regarded as secondary metabolites because the plant that manufactures them may have little need for them. They are naturally synthesized in all parts of the plant body; bark, leaves stem, root, flower, fruits, seeds, etc. i.e. any part of the plant body may contain active components (Tiwari *et al.,* 2011). Natural antioxidants or phytochemical are secondary metabolites of plants namely carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols, etc. are among the antioxidants produced by plants for their own sustenance. Beta-carotene, ascorbic acid and alpha tocopherols are widely used antioxidants. *Zingiber officinale* contains a number of antioxidants such as beta-carotene, ascorbic acid, terpenoids, alkaloids, and polyphenols such as flavonoids, flavones glycosides, rutin, etc. (Ghasemzadeh, 2010).

Z. officinale is one of the most widely used herbs and food flavouring agent.Zingiber comprising of 150 species and four sections distributed throughout tropical Asia, China, Japan and tropical Australia besides the subspecies (varieties): *Z. officinale var. rubra* and *Z. officinale var. rubrum* (Muda *et al.,* 2004). *Z. officinale* is known by different names in different parts of world. It is called different names in different part of the world e.g. Zenzero in Italian, Jeung or Sang Keong in Chinese, Aliah in Indonesia, Adrack in Urdu, Gember in Dutch, Jengibre in Spanish, Ingwar in German, Gingembre in French (Kumar *et al.,*2011).

In medicinal and aromatic plants, growth and biosynthesis of secondary metabolites are strongly influenced by genetic, environmental factors, and genetic×environmental effects (Pirbalouti *et al.,* 2014). Plant physiologists have been looking for new alternatives to conventional methods for improvimg in production of secondary metabolites. One of these methods is elicitation, which it can be an important strategy towards obtaining improved production of bioactive compounds (Bajalan *et al.,* 2017). Some chemical compounds that could be used as elicitors to modify secondary metabolites and subsequentlythe bioactivity of medicinal and aromatic plants. Recent years, the applications of signal components as elicitors have evolved an effective strategy for the production of target secondary metabolites in plant (Pirbalouti *et al.,* 2014).

MATERIALS AND METHODS

The study was conducted in rain-protected green house maintained. *Zingiber officinale* cv-*varada* was used for the present study. Ginger seed rhizomes were collected from Indian Institute of Spices Research, Kozhikode (ICAR Unit- IISR Kozhikode) and maintained in the greenhouse of Department of Botany, St. Joseph's College, Devagiri, Kozhikode, Kerala at 28^0C and the experimental area lies between 11.2588° N, 75.7804° E. Rhizomes were grown in grow bags containing soil and coco peat in the ratio 2:1. p^H of the soil 5.66, electrical conductivity 0.0302 S/m. There were about 15 grow bags kept for every single stress treatment.

Stress Treatments

When the ginger seedlings were at the second leaf stage, (approximately 90 days after planting) they were sprayed with two concentrations (10^{-2} and 10^{-3} M) of salicylic acid solution (SA; 2-hydroxybenzoic acid + 100 µl dimethyl sulfoxide + 0.02% Polyoxyethylenesorbitan monolaurate, Tween 20, Sigma Chemicals; pH 6.5), Zinc sulphate with same molar concentrations ($ZnSO_4$ 7 H_2O dissolved in distilled water + 0.02% Polyoxyethylenesorbitan monolaurate, Tween 20, Sigma Chemicals; pH 4). Control plants were sprayed with same solution but without SA and $ZnSO_4$. A trial was also maintained without any foliar application to estimate the amount of non volatile components of the variety under study. Apart from foliar spray drought stress is imposed by withholding water application, at the trials. Plants were sprayed once on the leaves early in the morning and every week until one month.

Morphological Analysis

Five plants/ treatments / replication were collected at random and studied for growth characters. Performance of ginger varieties for growth attributes like plant height, numbers of leaves per plant, number of tillers per plant were evaluated after 180 days of planting and yield attributes like weight of fresh rhizome were calculated at the end of harvest and the data was recorded.

Preparation of Extracts

Five grams of the dry powder from rhizome were weighed carefully and done hot methanolic extraction. The extracts were kept for 72 hours and then it is made up to 50ml with methanol.

Phytochemical Analysis

Estimation of antioxidant activity (DPPH Assay)

The antioxidant capacity of the extracts from ginger under various treatments was evaluated by DPPH assay (Hung *et al.,* 2005). Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. The amount of sample necessary to decrease the absorbance of DPPH (Sigma–Aldrich Co., Steineheim, Germany)

Estimation of total phenolics

The total phenolic content in the extracts was determined by theFolin–Ciocalteu (Sigma–Aldrich Co., Steineheim, Germany) assay (Singleton and Rossi, 1965). The absorbance of the samples was measured at 650 nm against a reagent blank using a UV–vis spectrophotometer (Thermoscientific Genesys 50). Gallic acid (Merck Co., Darmstadt, Germany) equivalent (GAL) was used as the reference.

Estimation of total flavonoids

Total flavonoids content in the extracts from sage under various treatments was determined according. Ordonez *et al.*, (2006) based on the formation of a flavonoid–aluminum complex with a maximum absorptivity at 510 nm. The flavonoids content is expressed as mg quercetin (Roth Co., Karlsruhe, Germany) equivalents per gram of each extract on dry basis mg quercetin/100g dry weight.

RESULTS AND DISCUSSION

Single variety of ginger (*Zingiber officinale* cv-*varada*) under various solitary as well as combined stress treatments were evaluated for variation in morphological as well as in preliminary phytochemical analysis. Morphological parameters under study include Plant height, Number of leaves per plant, Number of tillers per plant and yield attributes characterized by fresh and dry weight of the rhizome.

Comparing with the other treatments vegetative growth was found to be more prominent in plants sprayed with Salicylic acid. Spraying with 10^{-2} Molar Salicylic acid showed significant effect on number of leaves, height of the plant, fresh and dry weight of the rhizome than 10^{-3} Molar Salicylic acid. Increasing concentration of salicylic acid has a positive effect on morphological parameters (Table 1 & 2)

A study on *Thymus vulgaris* by (Khalil *et al.,* 2018) showed that decreasing field capacity and increasing SA concentration had a significant effect on height and fresh and dry weights of the plant compared to control plants. It was observed that foliar application of SA reversed the negative impact of drought stress on these three parameters at up to 2mM concentration.

Total Phenolics and Flavonoids

A standard gallic acid curve is prepared for the estimation of total phenolics, and standard quercetin curve is also prepared for the analysis of total flavonoids (Table 3 & Fig. 1).From the standard graph, total phenolics for different trials were calculated and recorded. A linear graph obtained with R^2 0.992 (Table 4).

Phenolic compounds contribute directly for antioxidative action and they constitute the main class of natural antioxidants present in the herbs (Awika *et al.,* 2003), therefore it is necessary to calculate amounts of phenolic compounds under different conditions. From the results it can be inferred that increasing concentration of foliar spray of salicylic acid has a positive impact on the total phenolic content when comparing with the foliar application zinc sulphate of same concentration.

Results of the present study showed significant differences in contents of total phenolic and flavonoid of the methanolic extract of ginger powder under different treatments (Table 3). The highest amounts of total phenolic of the extracts were obtained from combined stress treatments. Similarly, apart from solitary application of zinc sulphate and salicyilic acid combined with drought enhanced the total phenolics content. Bistgani *et al,.* (2017a) reported that mild drought stress caused an increase in amount of phenolic compounds in *T. daenensis*. In addition, results a study by Manukyan (2011) indicated that drought stress affected positively polyphenolic content in *Melissa officinalis* L. In general, water deficit increases polyphenolic content of more herbs, because in case of stress, more metabolites are produced in the plants and substances prevent from oxidization in the cells. On the other hand, some researchers have observed quite the opposite effect. For example, Bistgani *et al.,* (2017b) reported that drought stress inhibits the total synthesis of flavonoid in *T. daenensis* leaves and shoots.

The present study showed that the extracts from ginger rhizome under various stress condition had higher total phenolic contents under different stress combinations than individual treatments, indicating further that different levels of zinc sulphate and salicylic acid along with drought significantly affected the total phenolic content of the extracts. A significant interaction between irrigation frequencies and foliar application was found on total phenol and flavonoid contents. The maximum total phenolic content was obtained from the extract by the foliar spray of zinc sulphate and salicylic acid (10^{-2} Molar each) (Table 3). Jozeph *et al.* (2010) reported salicylic acid at concentrations 50 and 250 microM, for a period of up to 7 days significantly increased the content of polyphenols in M*atricaria chamomilla*. The exogenous application of salicylic acid also elevated the toatal phenolic content in table grapes (Blanch *et al.,* 2020). In response to changing ecological conditions, plants evolved the capacity to biosynthesize different phenolic component (Caldwell *et al.,* 2007). Quercetin standard graph was prepared for different concentration (Table 5 & Fig. 2). From the standard graph, total flavonoids for different trials were calculated and recorded. A linear straight line graph obtained with R^2 0.982 (Table 6).

The same trend is observed in the case of phenolics is followed here also. Combination of stresses resulted in higher flavonoid content. Here also foliar spray of salicylic acid shows a greater impact on the concentration of flavonoids than that of the foliar application of zinc sulphate. The study by Hassanpouraghdam, *et al.,* (2019) showed that, there exist a positive effect on flavonoids by foliar spray zinc and iron on *Rosmarinus officinalis.* Combined effect of CO_2 enrichment and foliar application of salicylic acid on the production and antioxidant activities of anthocyanin, flavonoids and isoflavonoids from ginger was studied by Ghasemzadeh *et al.,* (2012).

Antioxidant Assay by Dpph

The DPPH free radical scavenging activity is one of those indicators which are important in determining antioxidant potential of selected bioactive molecules or extract containing them. Antioxidant activities of the extract were evaluated using DPPH and represented in the table. Results of this study for free radical scavenging activity are statistically significantly different among different treatments (Table 6). The highest antioxidant activity of the extracts of sage was obtained from reduced irrigation with foliar spray of salicylic acid at high concentration treatment by DPPH assay (Table 6). These results can be attributed to the higher levels of polyphenolic (total phenol and flavonoid) in comparison to the well-watered plants. The results suggested that the phenolic components contributed significantly to the antioxidant capacity of the medicinal and aromatic plants (Pirbalouti *et al.,* 2014). Several investigations (Proteggente *et al.,* 2002; Corral-Aguayo *et al.,* 2008; Bajalan *et al.,* 2017) have studied correlations between bioactive compounds and antioxidant activity by DPPH assay in numerous herbs and spices. Literature review shows the presence of the two main groups of secondary metabolites in the genus of Salvia, volatile terpenoids and polyphenolic compounds (Roby *et al.,* 2013; Martins *et al.,* 2015) which are mainly responsible for the biological effects of the genus, particularly antioxidant activities. Interaction effects of foliar spray of salicylic acid×irrigation frequencies significantly influenced on the antioxidant activities by DPPH assay of the extracts (Table 4). The highest antioxidant activities in plants sprayed with 10^{-2} Molar salicylic acid under reduced irrigation condition (Table 6). Probably, Salicylic acid could regulate the activities of antioxidant enzymes and increase plant tolerance to abiotic stresses. Phenolic compounds are believed to account for a major portion of the antioxidant capacity in many plants (Yogesh *et al.,* 2012b). Epidemio-logical studies suggest that the consumption of flavonoid-rich foods protects against human diseases associated with oxidative stress. In vitro, flavonoids from several plant sources have shown free-radicalscavenging activity and protection against oxidative stress (Pirbalouti *et al.,* 2014b). At the same time foliar spray of zinc sulphate also had positive effect on the antioxidant activity. From the table it can also be inferred that the combination of stresses had a major impact on all the properties under study. Thus this crosstalk between different stresses is main reason for the increased amount of phenolics, flavonoids and antioxidant under study.

DPPH Assay

The radical scavenging effect of 15 samples were calculated and listed in table 7.

Table-1 Performance of ginger variety (*Zingiber officinale* cv-varada) for growth attributes (After 180 days of planting)

Treatments	Height of the plant(cm)	No.of leaves per plant	No.of tillers per plant
SA 10^{-2}molar	90.2	14.33	3.5
SA 10^{-3} molar	84.2	14.1	2
SA 10^{-2}molar+ drought	86.3	13	2
SA 10^{-3} molar+ drought	85	13.21	1
$ZnSo_4$ (C)	72.8	10.7	2
$ZnSo_4$ 10^{-2}molar	73.7	10.8	1
$ZnSo_4$ 10^{-3} molar	76.8	11	1
$ZnSo_4$ 10^{-2}Molar+ drought	77	12	1
$ZnSo_4$ 10^{-3} molar+ drought	78	13.5	2
SA 10^{-3} molar +$ZnSo_4$ 10^{-3} molar	75	12.8	1
SA 10^{-2}molar +$ZnSo_4$ 10^{-2}molar	85	13.5	1
SA 10^{-3} molar+ $ZnSo_4$ 10^{-2} molar	84	12.9	1
	82	11.2	1

Table-2 Performance of ginger variety (*Zingiber officinale* cv-varada) for yield attributes (After 180 days of planting)

Treatments	Rhizome fresh weight (in g)	Rhizome dry weight (in g)
Control	275	44.15
SA 10^{-2}molar	435	60.484
SA 10^{-3} molar	400	55.9
SA 10^{-2}molar+ drought	305	51
SA 10^{-3} molar+ drought	280	48.7
$ZnSO_4$ 10^{-2}molar	355	52
$ZnSO_4$ 10^{-3} molar	275	47
$ZnSO_4$ 10^{-2}molar+ drought	210	40.4
$ZnSO_4$ 10^{-3} molar+ drought	270	42.1
SA 10^{-3} molar +$ZnSO_4$ 10^{-3} molar	245	43
SA 10^{-2}molar +$ZnSO_4$ 10^{-2}molar	260	47
SA 10^{-3} molar+ $ZnSO_4$ 10^{-2} molar	256	45.2
SA 10^{-2} molar+ $ZnSO_4$ 10^{-3} molar	258	45

Table 3 The absorbance of different concentration of GA at 650nm

Concentration of quercetin (μg/ml)	Absorbance at 510 nm
200	0.161
400	0.366
600	0.489
800	0.559
1000	0.820

Table-4 The absorbance of different samples and its corresponding concentration of total phenolics obtained from the standard graph

Treatments	**Absorbance (at 650nm)**	**GA equivalent (mg GA/g)**
Control	0.732	13.2881
SA 10^{-2}molar	1.7865	34.4605
SA 10^{-3} molar	1.0325	19.3214
SA 10^{-2}molar+ drought	1.2315	23.31692
SA 10^{-3} molar+ drought	1.2275	23.23661
$ZnSO_4$ 10^{-2}molar	0.8915	16.4905
$ZnSO_4$ 10^{-3} molar	0.761	13.87035
$ZnSO_4$ 10^{-2}molar+ drought	1.3905	26.50928
$ZnSO_4$ 10^{-3} molar+ drought	1.469	28.08539
SA 10^{-3} molar +$ZnSO_4$ 10^{-3} molar	1.8005	34.74115
SA 10^{-2}molar +$ZnSO_4$ 10^{-2}molar	2.268	44.12749
SA 10^{-3} molar+ $ZnSO_4$ 10^{-2} molar	1.9585	37.9143
SA 10^{-2} molar+ $ZnSO_4$ 10^{-3} molar	2.089	40.5335

Table-5 showing the absorbance of different concentration of Quercetin at 510nm

Concentration of GA (μg/ml)	Absorbance at 650nm
5	0.228
10	0.685
20	1.099
30	1.498
40	2.10
50	2.531

Table-6 Showing the absorbance of different samples and its corresponding concentration of total Flavonoids obtained from the standard graph

Treatments	**Absorbance (at 510nm)**	**Quercetin equivalent (mg Q/g)**
Control	0.64	807.9709
SA 10^{-2}molar	0.74	933.25
SA 10^{-3} molar	0.66	833.027
SA 10^{-2}molar+ drought	0.78	983.368
SA 10^{-3} molar+ drought	0.75	945.78
$ZnSO_4$ 10^{-2}molar	0.64	807.9709
$ZnSO_4$ 10^{-3} molar	0.52	657.6
$ZnSO_4$ 10^{-2}molar+ drought	0.69	870.6127
$ZnSO_4$ 10^{-3} molar+ drought	0.65	820.4992
SA 10^{-3} molar +$ZnSO_4$ 10^{-3} molar	0.692	873.1183
SA 10^{-2}molar +$ZnSO_4$ 10^{-2}molar	0.77	970.83
SA 10^{-3} molar+ $ZnSO_4$ 10^{-2} molar	0.69	870.6127
SA 10^{-2} molar+ $ZnSO_4$ 10^{-3} molar	0.65	820.4992

Table-7 Showing the absorbance of different samples and its corresponding Percentage inhibition calculated at 517 nm

Treatments	Absorbance (at 650nm)	GA equivalent (mg GA/g)
Control	0.732	13.2881
SA 10^{-2}molar	1.7865	34.4605
SA 10^{-3} molar	1.0325	19.3214
SA 10^{-2}molar+ drought	1.2315	23.31692
SA 10^{-3} molar+ drought	1.2275	23.23661
$ZnSO_4$ 10^{-2}molar	0.8915	16.4905
$ZnSO_4$ 10^{-3} molar	0.761	13.87035
$ZnSO_4$ 10^{-2}molar+ drought	1.3905	26.50928
$ZnSO_4$ 10^{-3} molar+ drought	1.469	28.08539
SA 10^{-3} molar +$ZnSO_4$ 10^{-3} molar	1.8005	34.74115
SA 10^{-2}molar +$ZnSO_4$ 10^{-2}molar	2.268	44.12749
SA 10^{-3} molar+ $ZnSO_4$ 10^{-2} molar	1.9585	37.9143
SA 10^{-2} molar+ $ZnSO_4$ 10^{-3} molar	2.089	40.5335

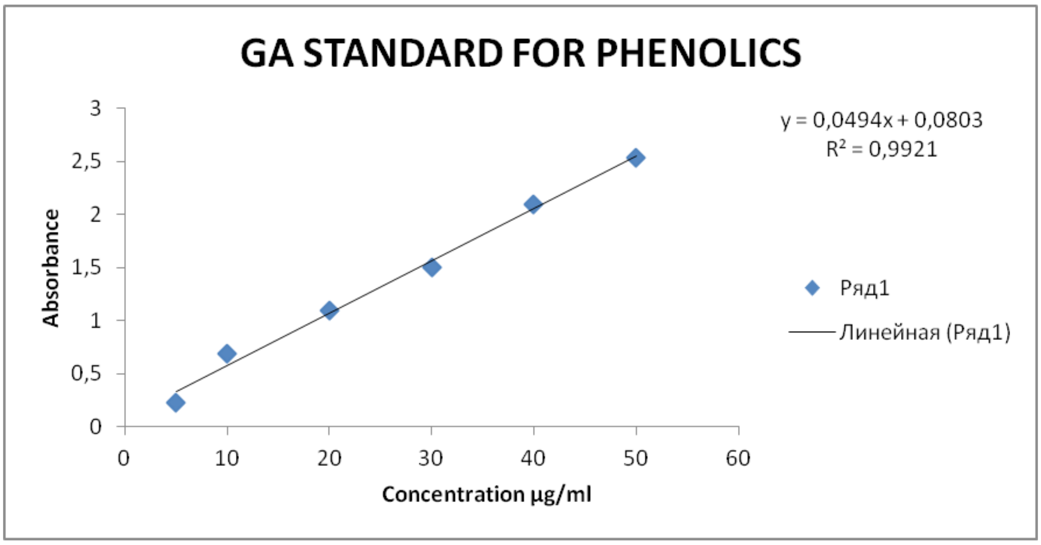

Figure 1. GA standard Graph for Phenolics

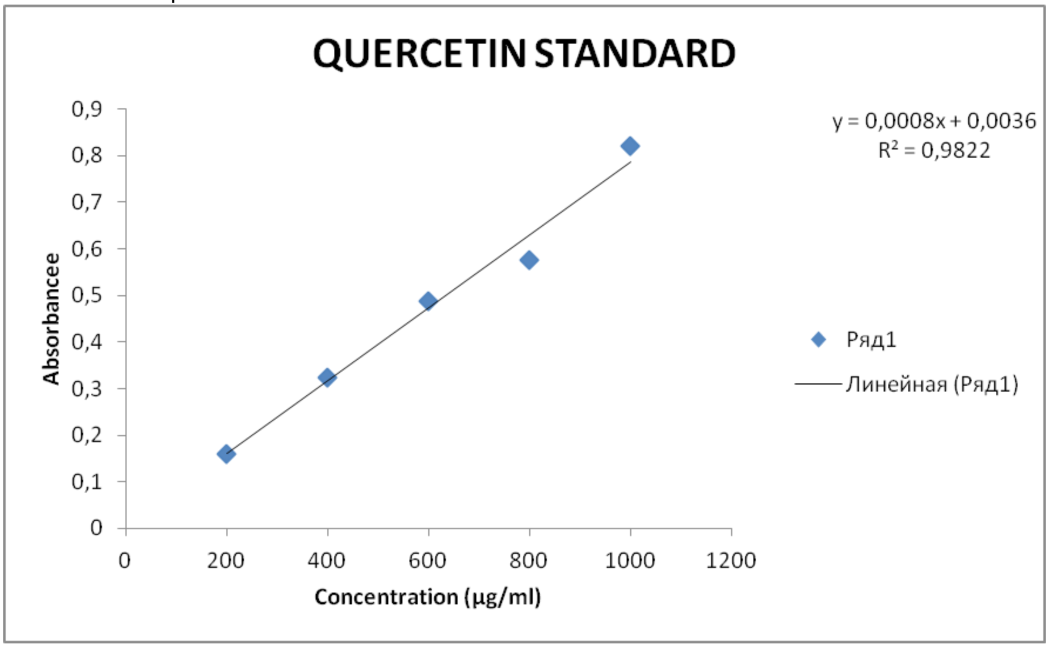

Figure 2. Quercetin standard graph for Flavonoids

CONCLUSIONS

This investigation demonstrated that stressful conditions stimulated the synthesis of secondary metabolites. Aerial spraying with SA and $ZnSO_4$ compensated to some degree for the negative impacts of reduced irrigation on production of some secondary metabolites total phenolic and flavonoid in the extract. The trials which shown maximum antioxidant activity are those with high amount of total phenolics. From these results, it is believed that SA is modifying the activity of enzymes responsible for the bioformation of ginger phenolics. In this sense phenyl-alanine lyase, which is the first enzyme regulating the phenylpropanoid pathway has been already reported to be affected by phytoregulators. As a result, the effect of SA on phenylalanine lyase activity would eventually result in an alteration of the bioformation of phenolics.

From the given study it could also be inferred that there occurs a crosstalk between the stress signals which could be the possible reason for the difference in the total phenolics, total flavonoids and antioxidant activity. From the results it can be inferred that increasing concentration of foliar spray of salicylic acid has a positive impact on the total phenolic content when comparing with the foliar application zinc sulphate of same concentration.

The present study suggests that *Z. officinale* for further research on improving the quality and phytochemical value of ginger by using various elicitors. This also provides a greater opportunity in studying and analyzing the molecular mechanism and the signal transduction pathway and how one signal interfere (could be both positively or negatively) with the transduction pathway or another signal. After all, Ginger is one of the major spice, for which drought is considered as a major menace, can be effectively overcome by this cross talk between different stress signals.

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CONFLICTS OF INTEREST

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

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