

Comprehensive Metabolite Profiling of Germinating Soybean (*Glycine max*) Seeds under Combined Abiotic and Biotic Stress by Gas Chromatography-Mass Spectroscopy

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ABSTRACT

Agriculture in India predominantly relies on the monsoon. After seed sowing, if no rain occurs for 8 to 10 days, it results in a brief drought. During the seed germination process, this climatic condition induces fungal infection in the seeds. This results in seed determination and farmers need to perform resowing. In this study, soybean seeds were subjected to drought, fungi (*Aspergillus flavus*), and combined drought-fungi stress treatments for 24 and 96 hours. The secondary metabolites were extracted in 80% methanol after stress treatment, and the samples were analyzed using gas chromatography-mass spectroscopy (GC-MS). Differential profile of secondary metabolites observed in various stress conditions up to 96 hours. On the first day 2,3, butanediol was found in drought, fungi and combined drought-fungi stress conditions. Methyl-6-(1 Methylpropyl)-galactopyranoside was observed in drought and combined drought-fungi stress conditions for 96 hours. The molecules observed in such stress conditions have antibacterial, antifungal, antiviral, and antioxidant activities, which shield the plant in various stress conditions.

Keywords: Abiotic and biotic stress, Combined drought-fungi stress, Soybean (*Glycine max*), GC-MS

Highlights

- Soybean seeds were exposed to drought, fungi and combined drought-fungi stress.
- Secondary metabolites were extracted and analyzed by GC-MS
- GC-MS analysis indicated the existence of 11 bioactive chemicals, demonstrating germicidal, fungicidal, and antioxidant action in maintaining soybean seeds under stressful circumstances.
- 2,3 Butanediol and Methyl-6-(1 Methylpropyl)-galactopyranoside signal their expression under coupled drought-fungi stress.
- The obtained metabolites play a vital role in protecting the plants under stress condition.s

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INTRODUCTION

With repeated exposure to several stresses at once, plants have emerged to survive in such environments. As a result of their sessile lifestyle, they have evolved specialized systems to recognize and react to complex stress situations and environmental changes, limiting damage while protecting vital resources for development and reproduction (Atkinson and Urwin 2012). A specific and distinct stress response is triggered in plants when they are exposed to many stresses at once (Deshmukh *et al.*, 2014).

Plant stressors can be categorized as either biotic (produced by pathogenic bacteria, fungi, nematodes, insects, or herbivores) or abiotic (caused by extreme droughts, flood, salt, temperatures, UV radiation, wounds, or heavy metals) (Piasecka *et al.*, 2019). Both biotic and abiotic stresses contribute significantly to the economic losses incurred by agriculture worldwide as a result of poorer yields (Nicol *et al.*, 2011).

It is well-recognized that salinity, high and low temperatures, and drought may affect the occurrence and spread of weeds, insects, and illnesses. Two stress combinations that are important to agriculture are heat-pathogen and drought-pathogen combos (Pandey *et al.*, 2017). Pathogen infection and drought stress interact both favorably and unfavorably. This combination of stresses has one of the most significant effects on crop yields globally, as evidenced by the volume of records of

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plant diseases impacted by drought stress and the frequency of drought stress occurrences (Arya *et al.*, 2021; Rajeb *et al.*, 2014).

Agriculture in India is mainly dependent on the monsoon. It is common for the rains to begin and the farmer to seed, but if there is no rain for eight to ten days, it results in a brief drought. This condition causes fungal infection in seeds during the germination process. If the seeds deteriorate, the farmer must sow them again.

Temperature variations and erratic rainfall might affect crop nutritional quality and production (Porter and Semenov 2005). The climate crisis will also have an impact on pest and pathogen

habitat ranges. Rising temperatures are favoring pathogen spread (Luck *et al.*, 2011). Crop plants are thus more prone towards various environmental stresses, which can have serious repercussions when they occur concurrently. There is an unparalleled need for crop types that can withstand stress owing to the changing climate and the increasing impact of population expansion on planetary food production (Newton *et al.*, 2011). Thus, it is essential to comprehend the processes behind plant responses to several concurrent stresses in order to facilitate the creation of wide-ranging stress-tolerant crops (Atkinson and Urwin 2012).

The current study focuses on soybeans (*Glycine max*), the third-most important leguminous commodity and one of India's most significant crops. It is a key cash crop that provides oil and proteins to humans, cattle, and aquaculture. Abiotic stressors that hinder plant development and lower worldwide soybean yield include drought, floods, and salinity.

Plant stress research is currently in dire need of focus in order to better recognize the nature of the different stress reciprocation exhibited by plants and to pave the way for the development of plants that are both highly productive and stress-resistant. Plants undergo physiological, molecular, and cellular modifications to cope with such harsh climatic circumstances. Abiotic and biotic stress conditions cause significant changes in the plant metabolome architecture. Therefore, the current research paper is concerned with the expression of secondary metabolites and their biological activities under drought, fungi and combined drought-fungi stress in germinating soybean seeds. To our awareness, this is an inaugural report in which we employed GC-MS to portray the metabolite profile in sprouting soybean seeds under combined drought-fungi stress. By applying this knowledge, we can develop a new soybean variety that is resistant to both drought and fungus assault. As a result, the farmer's yield of soybeans will grow while incurring no financial loss.

MATERIALS AND METHODS

Materials

We prepared every solution in distilled water using analytical grade and high-quality chemicals.

Plant material collection

We received the Soybean (KDS-726) seeds from Krishi Seva Kendra in Akola, Maharashtra. There were four groups created from the seeds. Healthy seeds were chosen, cleaned of dust with sterilized distilled water, and then submerged in 0.1% HgCl₂ solution for 10 minutes to disinfect the surface of the seeds.

Isolation of fungi

Fungi were collected from naturally infected soybean seed samples. To begin the isolation process, the infected soybean seed samples were soaked in sterilized distilled water, and then serial dilutions were made. About 0.1 mL aliquots of 10⁻⁶ dilution were cultured on 2.5% potato dextrose agar (PDA) plates. The inoculated plates were kept at room temperature in the laboratory for seven to ten days. As the fungi grew on each plate, sub-culturing was done on fresh plates, which were

then placed in an incubator set to a temperature of 27 to 29°C. Fungal growth was monitored daily, and the plates were then refrigerated at 4°C for further use (Czarnecka *et al.*, 2022).

Identification of fungal culture through Microscopy

After the isolation of fungal cultures from contaminated soybean seeds, a portion of the mycelial growth was taken with an aseptic inoculating loop and laid on a microscope slide containing droplet of lactophenol and the sample was spread, slide cover was placed and observed microscopically to identify spinning characteristics mycelium and spores (Pitt and Hocking 2009; Ahmed and Hadeel 2021).

Drought, Fungi and combined Drought-Fungi stress treatment

The sterilized soybean seeds were divided into two groups and exposed to stress. Seeds were deeped in distilled water for six hours in the first group and in the second group were immersed in the broth of an *Aspergillus flavus* culture flask for 6 hours. After that, seeds from group one were moved and divided into two new sets. For regulated conditions and routine watering, two layers of wet filter paper were used in the first set of clean, sterile Petri plates. In the second set, soaked seeds were placed on dried filter paper to simulate drought stress (Hackenberg *et al.*, 2015; Hivrale *et al.*, 2016).

The second group seeds that were exposed to the *A. flavus* culture was separated into two sets. In the first set seeds were placed on wet filter paper and regularly watered after being incubated in an *A. flavus* culture for six hours in order to create biotic stress (Lomte and Hivrale 2011). In the second set, *A. flavus*-activated seeds were spread out on dry filter paper for the combined treatment of drought and fungi stress. All four sets of seeds were individually collected after germination at 24 and 96 hours and kept at -80°C until needed. Three days old *A. flavus* cultures was used to infect seeds. Three times each experiment was conducted.

Germination analysis

Soybean seed germination was studied for 24 and 96 hours under control, drought, fungal, and combined drought-fungi stress conditions.

Preparation of sample

About 100 mg of soybean seeds were crushed in 1-mL of 80% methanol, followed by 10 minutes of sonication by 20 kHz probe and acoustic power 32.3 W then 10 minutes of centrifugation at 8000 rpm. The extract was prepared, supernatant was gathered, and it was passed through 0.45 µm syringe filter and the extract was hoarded in the dark at -20°C for additional investigation (Varela *et al.*, 2016; John *et al.*, 2017).

GC-MS Analysis

The analysis procedure was the same for each sample, but the oven temperature varied. The injection port temperature was 250, helium was the carrier gas moving at 1 ml per second, the interface was 250, and the ion source was 200. The evaluation was carried out using E+ IONISATION WITH 70ev, Accu TOF GCV, and HP-5 as the column through which the sample passes. MS

identification was finished in 36 minutes. NIST Ver. 2.0 library from the year 2005 was used for the detection.

In this separation and identification method, a combination analyzer that excels at both qualitative and quantitative analysis of organic compounds is gas chromatograph integrated with a high-resolution mass spectrometer. It transmits characteristics of high definition and precise mass measurement due to its smooth operation and immense sensitivity. Whilst a mass spectrometer is used to identify the separated components, a gas chromatograph is used for separation.

Statistical analysis

Every single sample was gathered and assessed in triplicate. The statistical evaluation was conducted using the MS Excel execution.

RESULTS AND DISCUSSION

Morphological and cultural characteristics of the fungal isolates

The fungal isolate was identified based on colony morphology as well as culture features. The oldest and most extensively used method for identifying fungi is to utilize morphological and cultural traits. *A. flavus* mycelium was originally white. After three days of incubation, the *A. flavus* colony generated olive-green conidia that predominated the colony's appearance. The colonies were flat near their boundaries and rose in the center. Green *A. flavus* colonies were seen, with conidia covering the top conidiophores.

Germination rate

Soybean seed germination rates varied significantly. For 24 and 96 hours, control seeds surpassed drought, fungus, and combined drought-fungi stress-treated seeds in terms of germination.

According to these findings, drought and fungal infection activate the defensive response, resulting in the synthesis of antifungal, antibacterial, antiviral, and antioxidant chemicals in developing seeds. Thus, the energy necessary for primary metabolism during normal seed germination is transferred to secondary metabolite production for their defense. Germination rates were reduced under stress conditions when compared to the control seed (Table 1). This result is consistent with those obtained by Lu *et al.*, (2021) and Hivrale *et al.*, (2016).

Identification of extracted secondary metabolites using GC-MS

The use of a gas chromatograph in conjunction with a mass spectrometer is a well-respected technology for analyzing phytochemical substances derived from natural sources. This

choice is supported by the inherent stability, sensitivity, and higher analytical efficiency of this analytical apparatus (Elghaffar *et al.*, 2022; Gera *et al.*, 2024). The result of this investigation affirmed the existence of various phytochemicals in methanol extract of control, drought, fungi, and combined drought-fungi stress-treated soybean seeds for 24 and 96 hours by GC-MS analysis.

Fig. 1a depicts the total four compounds found in the control set, i.e., soybean seeds germinated under regulated conditions for 24 hours using GCMS analysis. The four compounds found were acetic acid, 2,3 butanediol, tridecanoic acid methyl ester,

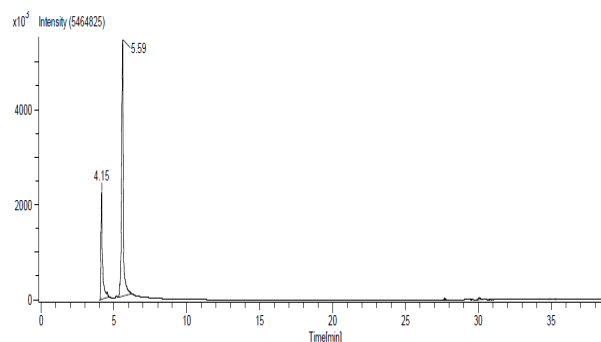


Fig 1: GCMS chromatograph of compounds identified in methanolic extract of germinated soybean seeds for 24 hours under a) control condition

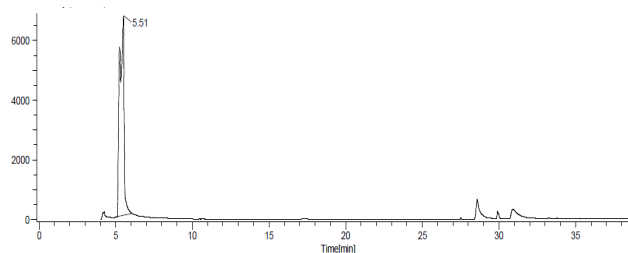


Fig 1 b): drought stress

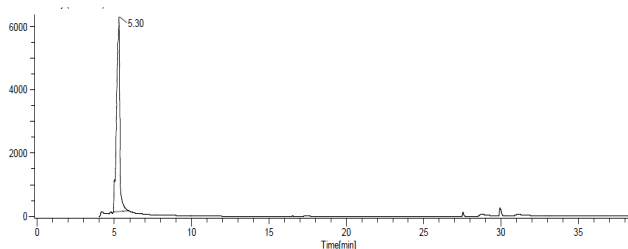


Fig 1 c): fungi stress

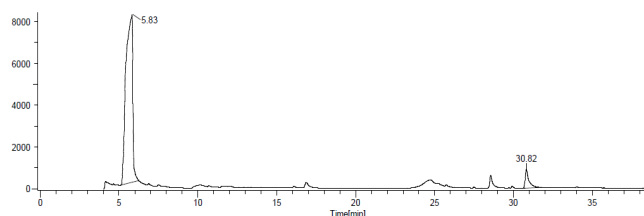


Fig 1 d): combined drought-fungi stress

Table 1: Effect of stress on germination rate

Stress	Control (%)	Drought (%)	Fungi (%)	Drought-fungi (%)
24 hours	80	00	00	00
96 hours	100	00	10	00

Table 2: The compound identified in the methanolic extract of germinating soybean seeds for 24 hours under a) control condition (GC-MS Study)

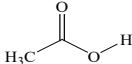

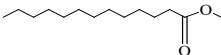
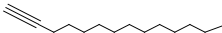
Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
Acetic acid		4.15	C ₂ H ₄ O ₂	60.00
2,3-butanediol		5.59	C ₄ H ₁₀ O ₂	90.00
Tridecanoic acid, methyl ester		27.69	C ₁₄ H ₂₈ O ₂	228.00
1-Tetradecyne		30.05	C ₁₄ H ₂₆	194.00

Table 2 b): Drought stress


Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
2,3-butanediol		5.49	C ₄ H ₁₀ O ₂	90.00

Table 2 c): fungi stress



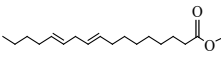
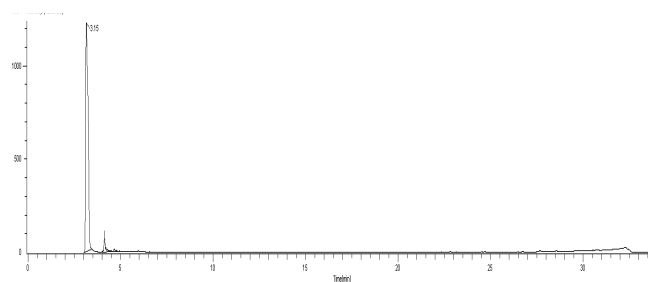
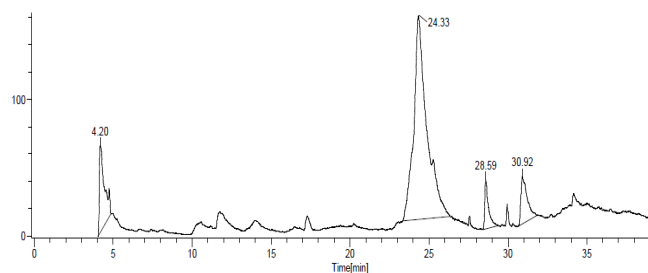
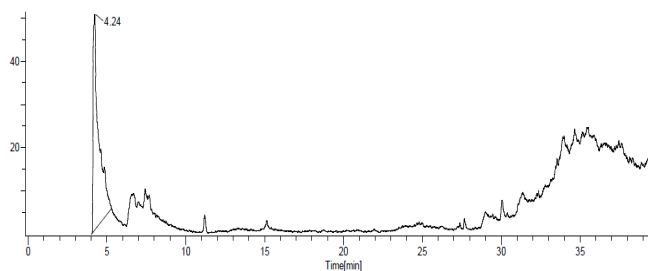
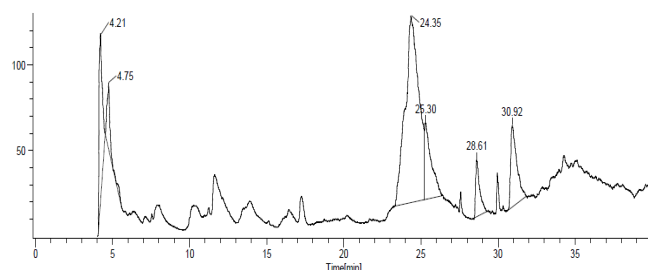
Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
2,3-butanediol		5.30	C ₄ H ₁₀ O ₂	90.00

Table 2 d): combined drought-fungi stress

Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
2,3-Butanediol		5.83	C ₄ H ₁₀ O ₂	90.00
9,12-Octadecadienoic acid		30.82	C ₁₈ H ₃₂ O ₂	280.00

and 1 tetradecyne (Table 2a).

According to Sun *et al.*, (2022) the exterior administration of acetic acid (AA) can boost drought resistance in perennial woody apple (*Malus domestica*) plants as well as drought existence in plants including cassava, rapeseed, rice, maize, wheat, and Arabidopsis. External application of acetic acid improved resistance to drought in apple trees by modulating abscisic acid (ABA) and jasmonic acid (JA)-induced mitogen-activated protein kinase (MAPK) signaling pathways. In addition, Amaechi 2021 observed that acetic acid has antibacterial and insecticide properties in *Moringa oleifera* (Table 4). 2,3-butanediol directly influences plant immunology and physiology, according to Hwe-Su Yi *et al.*, (2016). Moreover, it stimulated plant defense


Fig 2: GCMS chromatograph of compounds identified in methanolic extract of germinated soybean seeds for 96 hours under a) control condition

Fig 2b): drought stress

Fig 2c): Fungi stress

Fig 2d): Combined drought-fungi stress

in the roots, primarily through the ethylene and salicylic acid signaling pathways. In compliance with Song Mi Cho *et al.*, (2008) 2,3-butanediol had a significant role in developing drought resistance in Arabidopsis. 2,3 butanediol suppressed *R. solani* (fungal) growth in *A. stolonifera*, according to Yi *et al.*, (2018) and Huyan kong *et al.*, (2018) reported antiviral activity in pepper. According to Belakhader *et al.*, (2015) tridecanoic acid methyl ester exhibited microbicidal and fungicidal activities. Moreover, Elaiyaraja and Chandramohan (2018) reported activity against germs and fungi. About tetradecyne nothing has been reported.

GC-MS analysis of Soybean seeds germinated under drought and fungi stress conditions individually for 24 hours showed the presence of one compound (Fig. 1b and 1c). The compound was 2,3 butanediol (Table 2b and 2c).

Two compounds were obtained under combined drought-fungi stress conditions (Fig. 1d). The compounds were 2,3-butanediol and 9,12-octadecadienoic acid (Table 2d).

The antioxidant function of 9,12-octadecadienoic acid in *Abutilon theophrasti* Medic. Leaves were investigated by Chunlian *et al.*, (2018). The same research by Kim *et al.*, (2020) revealed that snow chrysanthemum and golden ring pumpkin pie contain 9,12 Octadecadienoic acid, which acts as an antioxidant. One common compound, 2,3 Butanediol, was present in the 24-hour-germinated soybean seeds under all three stress-treated samples (drought, fungi, and combined drought–fungi stress). 2,3- Butanediol reported to have antifungal activity by Tian *et al.*, (2023). It suggests that all three stress may share a common pathway for protecting the soybean seeds during the germination stage.

Fig. 2a depicts the total three compounds found in soybean seeds germinated under control conditions for 96 hours using GCMS analysis. The three compounds were hexane, hydrazine 1,2 dimethyl, and trifluoroacetyl-di-t-butylphosphine (Table 3a).

Kandasamy *et al.*, (2015) observed that Trifluoroacetyl-di-t-butylphosphine, was an antibacterial, antifungal, and antioxidant activity in *M. scabrella*. There is no information on the action of hexane and hydrazine 1,2 dimethyl.

From Fig. 2b, we found propanoic acid in drought-stressed seed samples, which has antioxidant and antibacterial properties, according to Sanjiv K. *et al.*, (2012). Azhagu (2021), found that methyl-6-(1 methylpropyl)-galactopyranoside acts as an antioxidant in the leaves of *Solanum torvum*. Undecanoic acid has significant antimycotic activity against *Aspergillus spp* as mentioned by Antonio *et al.*, (2021). Neepal *et al.*, (2019) uncovered antibacterial, nematicide and antioxidant activity of 9,12,15 Octadecatrienoic acid in *Cyperus alternifolius* (Table 3b). Capilla *et al.*, (2015) also proposed that 9,12,15 Octadecatrienoic acid (Linolenic acid) is the antecedent of Jasmonic acid, which mediates a new plant defense signalling route. Linolenic acid influences the expression of genes in reply to biotic and abiotic stressors.

Table 3: Components Detected in methanolic extract of germinating soybean seeds for 96 hours under a) control condition

Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
Hexane		3.15	C ₆ H ₁₄	86.00
Hydrazine 1,2-dimethyl		4.13	C ₂ H ₈ N ₂	60.00
Trifluoroacetyl-di-t-butylphosphine		4.66	C ₁₀ H ₁₈ F ₃ OP	242.00

Table 3b): drought stress

Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
Propanoic acid		4.20	C ₃ H ₄ O ₃	88.00
Methyl-6-(1-methylpropyl)-galactopyranoside		24.33	C ₁₁ H ₂₂ O ₆	250.00
Undecanoic acid		28.59	C ₁₁ H ₂₂ O ₂	186.00
9,12,15-octadecatrienoic acid		30.92	C ₂₂ H ₃₂ O ₄ Si ₂	496.00

Table 3c): Fungi stress

Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
2-Chloroaniline-5-sulfonic acid		4.24	C ₆ H ₆ ClNO ₃ S	207.00

Table 3d): Combined drought-fungi stress

Name of compound	Structure of compound	Retention time	Molecular formula	Molecular weight
Acetic anhydride		4.21	C ₄ H ₆ O ₃	102.00
1,2,4-Benzenetri carboxylic acid		4.75	C ₁₁ H ₁₀ O ₆	238.00
Methyl-6-(1-methylpropyl)-galactopyranoside		24.35	C ₁₁ H ₂₂ O ₆	250.00
Methyl-6-(1-methylpropyl)-galactopyranoside		25.30	C ₁₁ H ₂₂ O ₆	250.00
2-Cyclopentene-1-undecanoic acid		28.61	C ₁₆ H ₂₈ O ₂	252.00
2-Cyclopentene-1-undecanoic acid		30.92	C ₁₆ H ₂₈ O ₂	252.00

Table 4: Summary of reported biological activity of phytochemicals identified in the methanolic extract of germinating Soybean seeds under control, drought, fungi and combined drought-fungi stress condition for 24 and 96 hours

S. No	Name of the compound	Biological activity	References
1	Acetic Acid	Antibacterial, Antifungal, Insecticidal	Amaechi, 2021
2	2,3 butanediol	Antifungal, Antifungal	Song <i>et al.</i> , 2008; Yi <i>et al.</i> , 2018
3	Tridecanoic acid, methyl ester	Antibacterial, Antifungal	Belakhader <i>et al.</i> , 2015
4	Tetradecyne	Not reported	-
5	9,12 Octadecadienoic acid	Antioxidant	Chunlian <i>et al.</i> , 2018
6	Hexane	Not reported	-
7	Hydrazine 1,2 dimethyl	Not reported	-
8	Trifluoroacetyl-di-t-butylphosphine	Antibacterial, Antifungal, and Antioxidant	Kandasamy <i>et al.</i> , 2015
9	Propanoic acid	Antioxidant and Antibacterial	Sanjiv K. <i>et al.</i> , 2012
10	Methyl -6-(1-methylpropyl)-galactopyranoside	Antioxidant	Azhagu M. 2021
11	Undecanoic acid	Antifungal	Antonio <i>et al.</i> , 2021
12	9,12,15- Octadecatrienoic acid	Antimicrobial, Nematicide, Antioxidant	Neepal <i>et al.</i> , 2019
13	2-chloroaniline-5 sulfonic acid	Antibacterial	Muhammad <i>et al.</i> , 2014
14	Acetic anhydride	Not reported	-
15	1,2,4-Benzenetricarboxylic acid	Antifungal	Shailja K. <i>et al.</i> , 2022
16	2-Cyclopentene-1-undecanoic acid	Not reported	-

Under the fungi stress treatment, only one compound, 2-chloroaniline-5-sulfonic acid was obtained (Fig. 2c). Muhammad *et al.*, (2014) found that in the fungal-treated seed, 2 chloroaniline-5 sulfonic acid had antibacterial activity in *Paganum harmala* (Tables 3c and 4).

Shailja K. *et al.*, (2022) demonstrated antifungal activity of 1,2,4-benzenetricarboxylic acid in *Chaetomium globosum*. There is no information found on the action of acetic anhydride and 2-cyclopentene-1-undecanoic acid in plants (Fig. 2d and Table 3d).

CONCLUSION

Germination is essential to a plant's existence. Environmental stress, microbial attack, and nutritional depletion must all be dealt with by a growing seedling. In such a harsh environment, emerging seedlings fight itself opposed to abiotic and biotic stressors by such bioactive components *via* numerous metabolic cascades. The compounds examined by GC-MS exhibited antibacterial, antifungal, antiviral, insecticidal, and nematocidal activities.

Plants regularly create reactive oxygen species (ROS) as metabolic byproducts or signaling molecules in response to stress. During abiotic and biotic stress, ROS levels increase. Accelerated ROS result in oxidative damage to proteins, membrane lipids, and nucleic acids. To address this issue, components such as 9,12 octadecadienoic acid, trifluoroacetyl-di-t-butylphosphine, propanoic acid, methyl -6-(1-methylpropyl)-galactopyranoside, and 9,12,15-octadecatrienoic acid demonstrated antioxidant activity once more to protect the germinated seed under stress conditions.

Our finding will contribute significantly to the development of crops that can defy drought, fungi and combined drought-fungi stress. This information would be able to produce soybeans that are stress tolerant.

Farmers would benefit financially from this information, allowing them to produce more soybeans to fulfill the needs of the world's rising population.

In the future, we will be shedding light on plants' molecular responses to abiotic, biotic, and combinations of several distinct stressful circumstances.

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AUTHORS CONTRIBUTIONS

Ashwini K. Sirsat developed and carried out the experiment. Also collected the data, evaluated it, and authored the content for this research report. Heena L. Nadaf assisted in editing and revising the manuscript. Dr. Vandana K. Hivrale oversaw the conceptualization of this study report. Each author reviewed and gave their approval to the final draft.

CONFLICTS OF INTEREST

According to the authors, conflicts of interest don't exist.

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