

1 **Original Research Article**
2 **Defense priming in Okra using Chemically defined Elicitors**

3

4 **Abstract**

5 Biotic and abiotic stresses pose a threat to the growth and yield of plants. With increasing population and
6 decreasing viable land area, there is a dire need of stress-resistant crop plants. Bam-FX treatment provides
7 a sustainable method of priming the immune systems of plants without the need of genetic modifications.
8 Okra (*Abelmoschus esculentus*) seeds and seedlings were treated with chemically defined elicitors -Bam-
9 FX solutions of different concentrations to assess its effect on seed germination and plantlet
10 morphological growth. The gas chromatography-mass spectrometry (GCMS) and High resolution liquid
11 chromatography-mass spectrometry (HR-LCMS/MS) were used to assess various metabolites in treated
12 samples. We also treated some seeds with fungus (*Aspergillus* sp. 100 cfu/mL) or organic acids to assess
13 the priming effect in seeds.

14 We observed that, of the four Bam-FX concentrations initially selected, Bam-FX dilutions of 1:175,
15 1:250, 1:500 were most effective in inducing the most optimal seed germination and morphological
16 growth of the seedlings. Bam-FX treatment led to most marked upregulation of carboxylic acid and
17 fumaric acid in both Okra seeds and seedlings. The fungal infection seemed to exhibit synergistic effect
18 against Bam-FX treatment. The treatment with organic acids seemed to induce certain metabolites, such
19 as carbamic acid, phenylboronic acid, anthranilic acid, etc., that were not otherwise induced in the non-
20 Bam-FX-treated samples. Bam-FX, though originally designed for optimal plant growth in space,
21 exhibited a high potential in priming the defense mechanisms of Okra against abiotic stresses.

22 Keywords: Okra, Bam-FX, Biotic and abiotic stress, *Aspergillus* sp.

23 **Introduction**

24 Similar to animals, plants also suffer from several biotic and abiotic stresses. Such stresses adversely
25 affect the plant metabolism, which ultimately reduces their yield and productivity (Rejeb *et al*, 2014;

26 Shao *et al*, 2008). Abiotic stresses, such as unfavorable temperature, water deficit/excess, soil salinity,
27 etc., are known to affect the physiological reactions occurring inside the plant body, which drastically
28 reduces the plant growth and yield by more than 50% (Wang *et al*, 2003). Biotic stresses, such attack by
29 herbivores and pathogens lead to plant injury and diseases (Rejeb *et al*, 2014). Stress tolerance and
30 resistance is a crucial part of agriculture to enable better growth and yield of the crops and other useful
31 plants.

32 Several studies have shown that both abiotic and biotic stresses trigger the activation of several
33 transcription factors and effector proteins, and induce the accumulation of various types of secondary
34 metabolites that help protect the plant against such stresses (Atkinson and Urwin, 2012). In the past few
35 decades, several researchers have focused on “priming” of plant defense responses against different types
36 of biotic and abiotic stresses (Helms *et al*, 2017). Defense priming, using organismal and non-organismal
37 stimulants, can help in sustainable agricultural production by reducing the use of agrochemicals for the
38 protection of crops from pests and diseases. Prime defenses could be both physical and chemical, and
39 include morphological barriers, such as trichomes, cell wall lignification, and silica deposition, and
40 syntheses of toxic chemicals (alkaloids, terpenoids, and phenolics), which act as repellents, deterrents,
41 anti-nutritients, and anti-digestive compounds. Once primed, a plant defends itself more rapidly, strongly
42 and/or enduringly, against subsequent threats. Priming agents could be live organisms (e.g.
43 microorganisms or arthropods), chemicals (e.g. vitamins or plant hormones), or components thereof, and
44 can be applied to various tissues and at diverse developmental stages (for example, to foliage or roots of
45 mature plants, or to seeds). For instance, soaking of seeds in zinc or any other metal containing solution
46 for a specified time, after which the seeds are re-dried and sown, is a low-cost technique of seed
47 priming (Rehman *et al*, 2015). Zn priming of seeds, in turn, lead to a higher enhancement in vigor,
48 growth, and yield when compared to using zinc application techniques for soil (Montalvo *et al*, 2016).

49 Bam-FX® is a proprietary Elicitor composition developed by Zero Gravity Solutions Inc . It directly
50 affects the physiological mechanisms and innate immune system of plants to help them overcome both
51 abiotic and biotic stresses without the need for any form of genetic modification. It helps in reducing the

52 pest and microbial infestations and help in obtaining better yield even in regions with cold, heat, or water
53 stress. It enhances nutrient absorption, chlorophyll content, and antioxidant activity, and induces systemic
54 responses within the plant body.

55 In this study, We hypothesized that, not only will the priming treatment enhance the germination rate and
56 reduce the seed germination time, but will also promote the synthesis of different carboxylic acids as well
57 as the benzoic acid pathways.

58 **Methods**

59 **Bam-FX solutions and Okra seeds** 60 61

62 The standard Bam-FX solution was purchased from Zero Gravity Solutions Inc, USA, and diluted using
63 distilled water to obtain four concentrations of Bam-FX solutions, that is, 1:175 (least diluted), 1:250,
64 1:500, and 1:1000 (most diluted). The pH of the solutions was kept at 5.8 ± 0.2 . Intact Okra (*Abelmoschus*
65 *esculentus*) seeds were purchased from a local vendor.

66 *Treatment of pre-germinated seeds*

67 The Okra seeds were washed using sterile distilled water for 5 to 10 s. These seeds were then soaked in
68 the different diluted Bam-FX solutions for 5, 10, 20, 30, and 40 min at room temperature. After
69 incubation, the seeds were removed from the treatment solutions and aseptically placed on wet tissue
70 papers in tissue culture dishes. The culture dishes were then incubated at room temperature. After 12, 24,
71 48, and 72 h, seed germination and plantlet growth were recorded. The optimum exposure duration was
72 used for further experiments.

73 *Treatment of germinated seeds*

74 The Okra seeds were washed using sterile distilled water for 5 to 10 s and placed on wet tissue paper in
75 sterile tissue culture plates. The plates were then incubated at room temperature for 72 h. At this stage,
76 germinated sprouts were obtained. These sprouts were then treated with the different diluted Bam-FX
77 solutions for 5 min, 10 min, 20 min, 30 min, and 40 min at room temperature. After 12, 24, 48, and 72 h,

78 seed germination and plantlet growth were recorded. The optimum exposure duration was used for further
79 experiments.

80 *Fungal infection with Bam-FX treatment*

81 Seeds: The washed pre-germinated seeds were soaked with Bam-FX under optimal conditions, as
82 determined earlier. The treated seeds were placed on wet tissue paper in sterile tissue culture plates in two
83 sets. One set of seeds were infected with fungus (*Aspergillus* sp. 100 cfu/mL). All the seeds were
84 incubated at room temperature for 72 h.

85 Seedlings: The germinated sprouts were treated with Bam-FX under optimal conditions, as determined
86 earlier. The treated seeds were incubated for 24 h and subjected to metabolite extraction.

87 *Effect of organic acids on plant growth*

88 The washed Okra seeds were kept on sterile tissue paper in four different plates. Then, each plate was
89 filled with 1 mL of an organic acid. We used succinic, fumaric, benzoic, salicylic acids for our study. The
90 plates were then incubated at room temperature for 48 h. Next, the seeds were crushed and subjected to
91 cold methanolic extraction to obtain their metabolites.

93 *Metabolite extraction*

94 The pre-germinated (seeds) and germinated seeds (seedlings) were again treated under optimum
95 conditions (as determined above). The metabolites of treated samples were extracted using the hot and
96 cold methanolic extraction methods.

- 97 • Hot methanolic extraction: 10 mL methanol:water (1:1) was mixed with 1 g of crushed
98 seeds/seedlings and kept at 70 °C for 15 min. Then, the mixture was incubated for room
99 temperature and used for the preparation of esters.
- 100 • Cold methanolic extraction: 10 mL methanol was mixed with crushed seeds/seedlings and kept at
101 4 °C for 14 h. After incubation, the mixture was used for the ester preparation.
- 102 • Methyl ester preparation: 250 µg or 50 µL of lipid samples were added to 1 mL of 1% methanolic
103 NaOH (freshly prepared). The samples were heated at 55 °C for 15 min. In hot tubes, 2 ml of 5%

104 methanolic HCl (freshly prepared) was added and the mixture was again heated for 15 min at
105 55 °C. The fatty acid methyl esters (FAME) were eluted by adding 1 mL of hexane to the above
106 mixture. FAME were then stored at 8-10 °C.

107 *GCMS*

108 The methyl esters obtained from the samples (as described in previous subsection) were used for GCMS.
109 GC-MS analysis (Agilent 5975C gas chromatography system) was performed following the users' guide
110 to calibrate method with FAME standards, available on Fiehn GC-MS Metabolomics library-2008
111 (Agilent Chem Station, Agilent Technologies Inc., Wilmington, USA), with slight modifications, using
112 retention time locking method. HP-5MS capillary column (30 m length, 10 m Duraguard, 0.25mm
113 diameter; narrow bore and 0.25µm film) manufactured by Agilent J&W GC columns, USA, was used for
114 the analyses. Following oven temperature program was maintained: 60 °C (1 m), followed by 325°C at
115 10°C/min as final hold for 10 min before cool-down. Run time was 37.5 min. Injection temperature was
116 set at 250°C, MSD transfer line at 290°C, and ion source at 230 °C. Helium was used as carrier gas
117 (constant flow rate of 0.723 mL/min; carrier linear velocity 31.141 cm/s). Sample (1 µL) was injected
118 onto the column via split mode (split ratio was 1:5). Chromatograms were analyzed using Automated
119 Mass Spectral Deconvolution and Identification System (AMDIS). Metabolite identification was
120 performed by comparing the retention times (Rt), retention indices (RI), and mass spectral fragmentation
121 pattern of compounds using the references present in Agilent Fiehn Metabolomics library. Many of the
122 metabolites were also identified by comparing chromatographic and spectral properties with that of
123 standard compounds.

124 *HR LCMS/MS*

125 Metabolites were analyzed using a Vanquish UHPLC system (Thermo, USA) connected to a Q Exactive
126 Plus - Orbitrap MS (Thermo, USA). The system was operated in positive electrospray ionization mode.
127 Metabolites were separated on a C-18 reverse-phase column (2.1 × 150 mm, 1.8 µm particle size).The
128 auto-sampler and column temperature was kept constant at 4°C and 40°C, respectively. Mobile phase A
129 contained 0.1% formic acid in water and mobile phase B contained water . The flow rate was 300 µL/min.

130 Solvent B was held at 5% for 2 min, and then, increased to 95% between 2 and 20 min, lowered to 5%
131 from 20 to 25 min and held at 5% between 25 and 26 min. Run was stopped at 30 min. The limit of
132 detection was set at 3 times the signal-to-noise ratio, whereas the limit of quantitation was set at 10 times
133 the signal-to-noise ratio.

134 **Results**

135 Effect of Bam-FX on seed germination and seedling growth

136 The Okra pre-germinated (Fig:1) and germinated (Fig:2) seeds were treated with four different
137 concentrations of Bam-FX. As shown in Fig 1, $\geq 80\%$ germination rate and optimum root and leaf growth
138 was observed when the seeds were treated using solutions with 1:175 (10 min exposure) and 1:500 (30-40
139 min exposure) dilutions. On the other hand, in case of post-germinated seeds, the most optimum growth
140 of seedlings was observed when they were treated using solutions with 1:175 (10 min exposure) and
141 1:250 (5 min exposure) dilutions. Solution with lower concentration (1:1000 dilution) needed to be
142 exposed for longer durations (40 min or higher) to achieve acceptable germination and growth.

143 Effect of Bam-FX treatment on the carboxylic acid content

144 We observed that treatment with a concentrated solution of Bam-FX (1:175 dilution) led to an initial
145 increase in the carboxylic acid content in Okra seeds at 12 h, followed by a decrease at 24 h, compared to
146 the control samples. In contrast, treatment with a diluted solution (1:500 dilution) led a marked rise in the
147 carboxylic acid content at 24 h compared to the control samples. We obtained a 16.85% and 19.3%
148 carboxylic acid content at 24 h following hot and cold methanolic extraction methods, respectively
149 (Figure 1).

150 Analysis of secondary metabolites in Okra seeds using GCMS

151 As shown in Fig 2, for seeds treated with 1:175 Bam-FX, we observed an increase in the levels of 2,6-
152 dichloro-4-nitrophenol, succinic acid, 5-(Heptafluoropropyl)uridine, and cyclohexanepropanoic acid at 12
153 h, and tris(methoxydimethylsilyl)silane, 2,5,8,11,14,17-hexaoxaoctadecane, dimethyl 2-butyl-1-(2-

154 trimethylsilylethoxymethyl)imidazole-4,5-dicarboxylate at 24 h, compared to corresponding control
155 samples.

156 Furthermore, for seeds treated with 1:500 Bam-FX, we observed an increase in the levels of pentanoic
157 acid, ethyl homovanillate (TMS derivative), and cyclohexanepropanoic acid at 12 h, and
158 tris(methoxydimethylsilyl)silane and 3-ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane at 24
159 h, compared to corresponding control samples. When the same analysis was repeated following the cold
160 methanolic extraction method, at 24 h, we observed an increase in the levels of 2'-hydroxypropiophenone,
161 silicic acid, and dimethyl 2-butyl-1-(2-trimethylsilylethoxymethyl)imidazole-4,5-dicarboxylate, and
162 fumaric acid (eicosyl 2-methylpentyl ester) in seeds treated with 1:175 and 1:500 Bam-FX, respectively
163 (Fig 3).

164 Analysis of secondary metabolites in Okra seeds using LCMS

165 Next, we assessed the levels of seeds treated with 1:175 and 1:500 Bam-FX followed by incubation for 24
166 h . At 24 h, we observed an elevation in the levels of B glyceric acid, S 10 Hydroxycamptothecin,
167 erythromycin, turmerone, aspartic acid, taxifolin, and epimedin A after treatment with 1:175 Bam-FX,
168 and in levels of arachidonic acid, artemisinin, gedunin, ginsenosides, guanidosuccinic acid, picrotoxinin,
169 quinine, taxifolin, ricinine, and linoleic acid after treatment with 1:500 Bam-FX(Figures 2a and b).

170 Effect of BamFX on germinated Okra Seeds

171 In search of the effect of BamFX on seedlings , we used 48 hours grown untreated seedlings. These
172 seedlings were sprayed with BamFX dilutions 1:175 and 1:500. Samples were taken out at 12 hours and
173 24 hours after BamFX treatment. In these seedlings , we observed elevation in the levels of anthranilic
174 acid, propanoic acid, 4 nitrothalic acid, and octadecanoic acid, and at 24 h, we observed elevation in
175 levels of cyclohexaneacetic acid, 2,2,3,3,4,4,5,5,6,6,7,7-dodecafluoroheptylester, triazole carboxylic acid,
176 2-ketoisocaproic acid, dimethyl malonic acid, fumaric acid, and butanoic acid (Figure 3).

177 For seeds treated with 1:500 Bam-FX, at 12 h, we observed elevation in the levels of sebacic acid,
178 dimethylmalonic acid, butanoic acid, and silicic acid, and at 24 h, we observed elevation in levels of 2-
179 ketoisocaproic acid, fumaric acid, benzoic acid, and silicic acid (Figure 3).

180 Effect of fungal infection on secondary metabolites of Bam-FX-treated seeds

181 We observed an elevation in the levels of jervine, picrotoxinin, quinine, rescinnamine, ricinine, taxifolin,
182 linolenic acid and ascorbic acid in the Bam-FX treated seedlings infected with *Aspergillus* sp. (Figure 4a).
183 In case of seeds, we observed elevated levels of psoralidin, erythromycin, aconitine, gedunine,
184 ginsenosides, 18 B glycyrrhetic acid, arachidonic acid and aspartic acid (Figure 4b).

185 Effect of organic acid treatment on Bam-FX treated seeds

186 Bam-FX treated seeds were treated with succinic, fumaric, benzoic and salicylic acids and incubated for
187 24 h and 48 h after treatment. We assessed the metabolic profiles of the samples at both time points. We
188 observed that, after treatment with succinic acid, the levels of cyclamic acid, terephthalic acid,
189 phenylboronic acid, phenylacetic acid, 3,4 dihydroxymandelic acid, 1, Nitro, 9,10-dioxo-9-10-
190 dihydroanthracene-2-carboxylic acid, 1,3,5 benzenetricarboxylic acid were elevated at 24 h, and those of
191 silicic acid, bicyclooct-2-ene-2,3-dicarboxylic acid, 1,2,4benzenetricarboxylic acid, 5 methylsalic acid, 4-
192 isoquinoline carboxylic acid, and benzoic acid were elevated at 48 h (Figure 5a).

193 After treatment with salicylic acid, the levels of salicylic acid, undecanoic acid, carbamic acid, 4-(4,7-
194 dimethoxy-2H-1,3-benzodioxol-5-yl)-2-oxopyrrolidine-3-carboxylic acid, pyridine-3-carboxylic acid,
195 1,4-dihydro-5-cyano-2-hydroxy-4-(4-isopropylphenyl)-6-methyl-4H-pyran-3-carboxylic acid, 6-amino-5-
196 cyano-2-methyl-4-(4-pyridyl), 4,5-dibromohex-2-enoic acid were elevated at 24 h, and those of arsenous
197 acid, tartronic acid, acridin-9-yl-amino-acetic acid, and benzoic acid were elevated at 48 h (Figure 5b).

198 After treatment with fumaric acid, carbamic acid and 1,2-cinnolinedicarboxylic acid, 1,2,3,5,6,7,8,8a-
199 octahydro-4-trimethylsilyloxy-diethyl ester were induced at 24 h, while 18-norcholest-17(20),24-dien-21-
200 oic acid and 16-acetoxy-4,8,14-trimethyl-3,11-dioxo-, methyl ester were found at 48 h only (Figure 5c).

201 At both time points, we observed the generation of arsenous acid and silicic acid; however, their levels
202 were higher at 48 h compared to the levels at 24 h (Figure 5c).

203 In case of benzoic acid, phenylboronic acid, anthranilic acid, and dibenzoazepinmalonamic acid were
204 induced at 24 h, while pyridine-3-carboxylic acid, 1,4-dihydro-5-cyano-2-hydroxy-4-(4-isopropylphenyl)-
205 6-methyl-ethyl ester, 4-acetyloxyimino-6,6-dimethyl-3-methylsulfanyl-4,5,6,7-tetrahydro-

206 benzo[c]thiophene-1-carboxylic acid methyl ester, 3,4-dihydroxymandelic acid, 4TMS derivative,
207 terephthalic acid, 4-bromo-2,6-difluorobenzyl ethyl ester were found at 48 h only (Figure 5c). At both
208 time points, we observed the generation of silicic acid and 3,4-dimethylbenzoic acid, TBDMS derivative;
209 however, their levels were higher at 48 h compared to the levels at 24 h (Figure 5d).

210 **Discussion**

211
212
213 Environmental stresses, both biotic and abiotic, pose a major challenge in adequate growth and
214 development of plants. Such stresses ultimately lead to a decrease in the yield of the plants, including
215 agricultural crops and economical plants. The immune responses of plants to such stressors are extremely
216 complex and changes constantly based on the type of stress. Several chemical compounds, such as
217 fungicides, insecticides, and fertilizers, are used to promote better plant growth and yield and thwart the
218 attack of adverse elements, such as pests, harmful pathogens, and environmental stresses. A plethora of
219 signal transduction pathways and networks act and interact with each other to mediate the plant responses
220 against the different types of stresses (Peck and Mitler, 2020). An important part of plant defenses include
221 rapid stress perception and deployment of adequate counter-measures (Rejeb *et al.*, 2014). The basal
222 constitutive defense mechanisms of plants play an important role in the recognition of the type of stress
223 and activation of the corresponding signaling cascades (Andreasson and Ellis, 2010; Chinnusamy *et al.*,
224 2004). Each stress is countered with a specific type of signaling response. However, when facing more
225 than one type of stress, the plant's overall response cannot be predicted from its respective responses to
226 individual stresses (Atkinson and Urwin, 2012). Several researchers are still attempting to elucidate such
227 multiple responses of plants in both lab and field conditions. Furthermore, unlike animals, plants do not
228 possess a somatic immune system or mobile immunity cells (Jones and Dangl, 2006). Instead, plants are
229 dependent upon the innate immune system and the signaling molecules that are released by the cells
230 located in adversely affected areas on plant bodies (Ausubel FM, 2005; Jones and Dangl, 2006). The
231 innate immunity system of plants is broadly divided into two parts. One part of this immune system

232 employs the pattern-recognition receptor (PRRs), which are present on the cell surfaces and are used to
233 recognize the damage-associated molecular patterns (DAMPs) derived from the host body and microbe-
234 associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) that are
235 found in a wide range of pathogenic microbes (Han G-J, 2011; Boller and Felix, 2009). The other part
236 employs the disease resistance (R) proteins that act on the pathogen-released effector molecules that are
237 responsible for the infections (Upson *et al.*, 2018). Previous studies have shown that exposure to these
238 elicitors, such as DAMPs, PAMPs, chemical or physical stimulating agents, could aid in modification in
239 the expression of the “defensive genes” of plants. Such genes are generally involved in the induced
240 systemic resistance and systemic acquired resistance. The process of inducing such genes using artificial
241 elicitors is essentially termed as priming (Mauch-Mani *et al.*, 2017). Here, we used a proprietary chemical
242 cocktail mixture, Bam-FX, developed by Zero Gravity Solutions Inc. to induce defensive responses in
243 Okra.

244
245 The active ingredient in Bam-FX is zinc sulphate (6.9%) and copper sulphate pentahydrate (2.1%),
246 mixed with inert carriers (91%), containing water (86%) and other sulphates (5%). Its application helps in
247 the movement of necessary ions to deficiency sites, which, in turn, improve plant yield, growth, and
248 resistance against pests and pathogens. Although its efficiency has been proved in previous studies, the
249 mechanism of its action in different plant species and in various environmental and soil conditions has
250 not yet been completely elucidated. To the best of our knowledge, this is the first study elucidating the
251 effects of optimum concentrations of Bam-FX solution on Okra growth, yield, and metabolism.

252
253 Here, we observed that the application of Bam-FX promoted the seed germination and morphological
254 growth of Okra. However, we observed that both very low and high concentrations of Bam-FX were less
255 efficient than the concentrations in-between. Among all the concentrations, the Bam-FX solution with
256 1:175 dilution was found to be most effective in promoting the morphological traits of both pre- and post-
257 germinated Okra seeds.

258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283

Several studies have previously shown that, apart from being primary metabolites, carboxylic acids are potent priming agents in plants (Gamir et al, 2012; Mauch-Mani *et al*, 2017). Here, our results indicated an increase in the production of carboxylic acids in Okra seeds and seedlings following treatment with all the concentrations of Bam-FX. However, the highest synthesis of carboxylic acids was observed when Okra was treated with Bam-FX with 1:175 and 1:500 dilutions.

Organic acids are mainly a part of the Krebs cycle in mitochondria and, to some extent, the glyoxylate cycle in glyoxysomes. They are generally present in small amounts, but, under stress, their levels increase. Such increase in levels of organic acids is then encountered by plants via the production of several secondary metabolites, such as flavonoids and phenolic compounds (López-Bucio K *et al*, 2000). Our results indicated that exposure to organic acids led to an increased synthesis of such compounds in the plants treated with Bam-FX.

It is noteworthy that, to the best of our knowledge, this is the first study involving optimization of adequate solution concentration and duration of exposure to Bam-FX, and previous literature in this respect is highly limited. However, our results indicate the Bam-FX holds great potential in improving the growth and tolerance of the plants via natural mechanisms, hence reducing the need to use harmful fertilizers and pesticides and other alternative techniques, such as genetic manipulation. The main limitation of this study was that we did not compare the effects of Bam-FX with those of conventionally used fertilizers. In their recent attempt to establish a pest management program in tomato, Mauch-Mani *et al* (2017) reported that, although Bam-FX promoted the growth and yield of the plants, it was still less effective than the conventional N-P-K fertilizer. However, they reported better pest resistance in the plants treated with Bam-FX. These findings also warrant the need for further studies on the optimizing the use of Bam-FX on various plants under different soil conditions.

284 **COMPETING INTERESTS DISCLAIMER:**

285 Dr. Raveendran Pottathil is Chief Technology Officer and COO of Zero Gravity Solutions Inc. USA and
286 he invented a Bam-FX induced multigenerational priming in plants. The BamFx is a product of Zero
287 Gravity Solutions Inc. USA.

288 **References**

- 289
- 290 Andreasson E and Ellis B.(2010) Convergence and specificity in the Arabidopsis MAPK nexus.
291 Trends in plant science 15:106–113.
- 292 Atkinson N and Urwin P-E. (2012) The interaction of plant biotic and abiotic stresses: From genes to
293 the field. Journal of Experimental Botany 63:3523–3544.
- 294 Ausubel FM. (2005) Are innate immune signaling pathways in plants and animals conserved? Nature
295 Immunology 6:973–979.
- 296 Boller T and Felix G. (2009) A renaissance of elicitors: perception of microbe-associated molecular
297 patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology.
298 60:379–406.
- 299 Chinnusamy V, Schumaker K, Zhu JK. (2004) Molecular genetics perspectives on cross-talk
300 and specificity in abiotic stress signalling in plants. Journal of Experimental Botany 55:225–236.
- 301 Han G-Z. (2019) Origin and evolution of the plant immune system. New Phytologist 222: 70–83.
- 302 Helms AM, Moraes CM, Troger A, Alborn HT, Francke W, Tooker JF, et al. (2017) Identification of
303 an insect-produced olfactory cue that primes plant defenses. Nature Communications. 8:337.
- 304 Jones JDG and Dangl JL. (2006) The plant immune system. Nature. 444:323–329.
- 305 Lo´pez-Bucio K et al. (2000) Organic acid metabolism in plants: from adaptive physiology to
306 transgenic varieties for cultivation in extreme soils. Plant Science. 160:1-13.
- 307 Montalvo et al. (2016) Chapter Five - Agronomic Effectiveness of Zinc Sources as Micronutrient
308 Fertilizer. Advances in Agronomy 139:215–267.
- 309 Peck S and Mitler R. (2020) Plant signaling in biotic and abiotic stress. Journal of Experimental
310 Botany. 71:1649–1651.

311 Rehman et al. (2015) Seed priming with zinc improves the germination and early seedling growth of
 312 wheat. *Seed Science and Technology* 43:262–268.

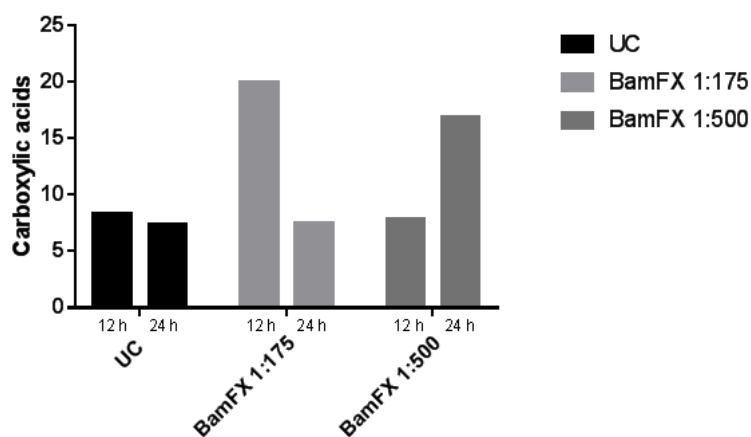
313 Rejeb BI, Pastor V, Mauch-Mani B. (2014) Plant Responses to Simultaneous Biotic and Abiotic
 314 Stress: Molecular Mechanisms. *Plants*. 3:458–475.

315 Shao HB, Chu L-Y, Jaleel CA, Zhao C-X. (2008) Water-deficit stress-Induced anatomical changes in
 316 higher plants. *Comptes Rendus Biologies* 331:215–225.

317 Upson JL et al. (2018) The coming of age of Evo MPMI: evolutionary molecular plant–microbe
 318 interactions across multiple timescales. *Current Opinion in Plant Biology* 44:108–116.

319 Wang W, Vinocur B, Altman A. (2003) Plant responses to drought; salinity and extremetemperatures:
 320 Towards genetic engineering for stress tolerance. *Planta*. 218:1–14.

321 Mauch-Mani B et al. (2017) Defense Priming: An Adaptive Part of Induced Resistance. *Annual*
 322 *review of plant biology*. 68:485–512.



323

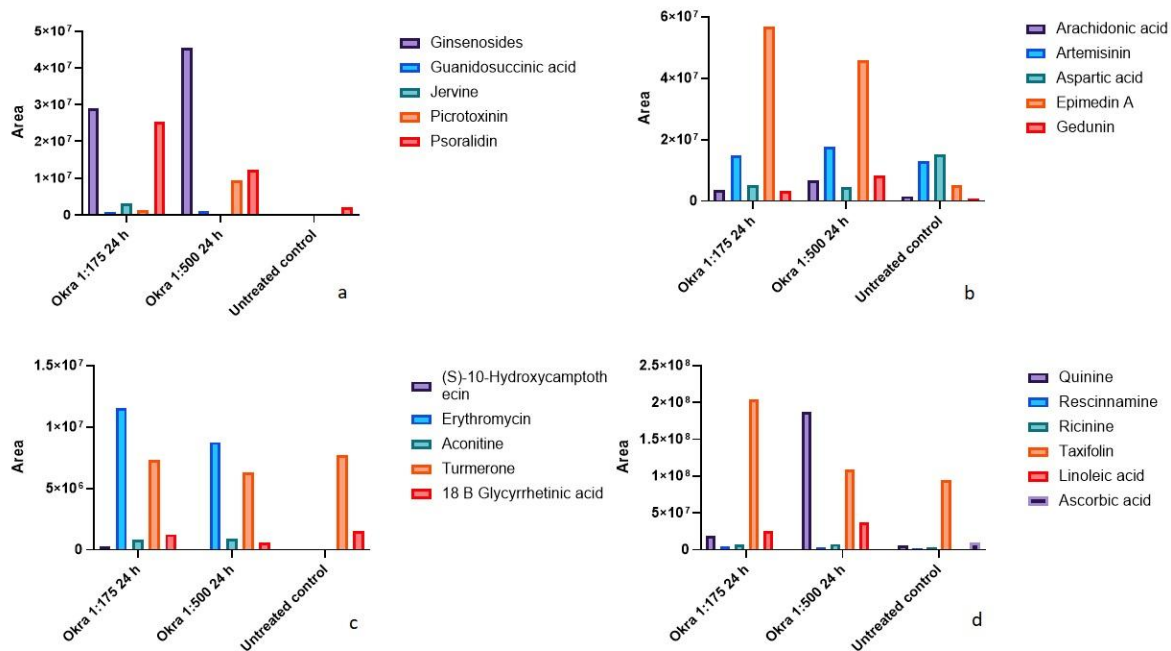
324 **Fig1. Carboxylic acid induction in Bam-FX treated Okra seeds.**

325 The UC – untreated seeds , BamFX1:175 – BamFX dilution in water 1:175 , BamFX 1:500 – BamFX

326 dilutions in water 1:500. The carboxylic acids were estimated after 12 h and 24 h in treated and untreated

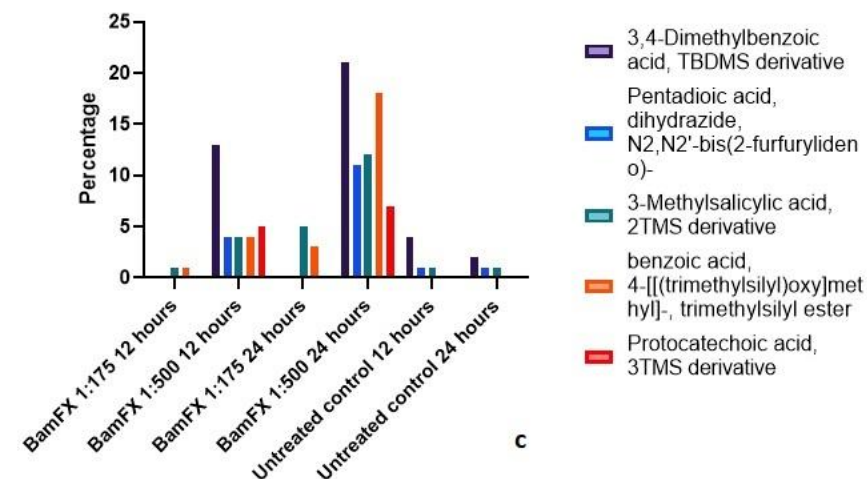
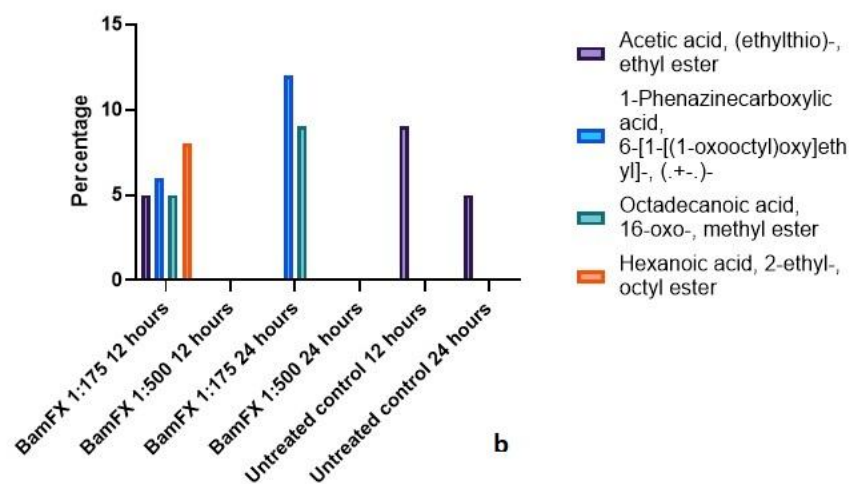
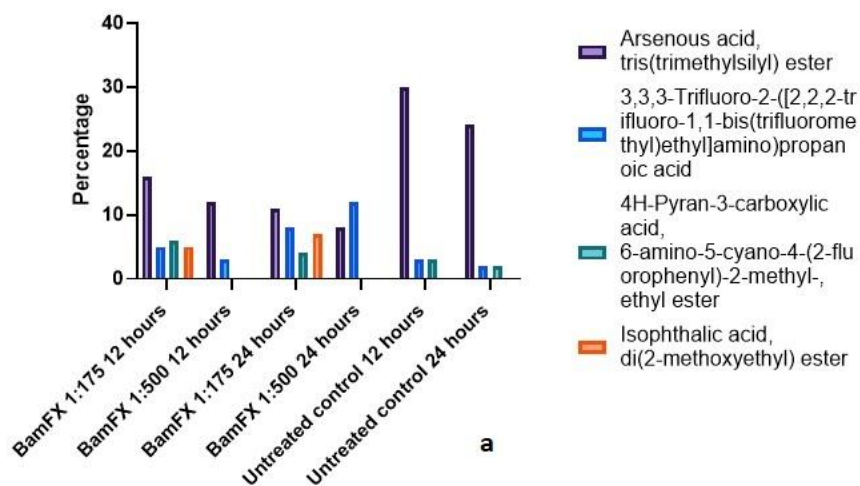
327 seeds.

328



329
330

331 **Fig 2. Enhancement of the carboxylic acid production in the seeds and seedlings treated with**
 332 **BamFX** . The HR LCMS/MS was used for the analysis of the Carboxylic acids in the samples. 1:175
 333 24 h- carboxylic acids extracted after 24 h from Seeds treated with BamFX dilution 1:175. 1:500, 24
 334 h- carboxylic acids extracted after 24 h from Seeds treated with BamFX dilution 1:500. 1:175,
 335



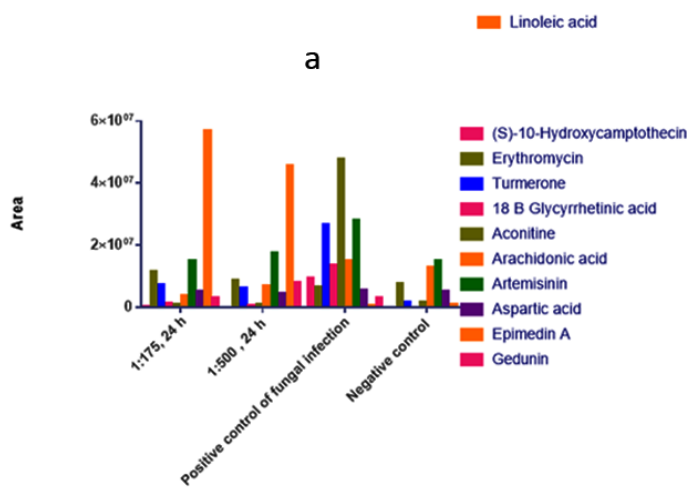
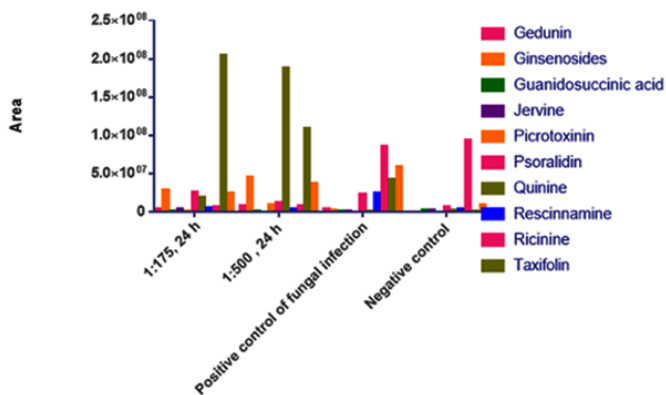
336

337 **Fig 3. Induction of carboxylic acid in seedlings treated with the BamFX.**

338 *Untreated control 12 hours- untreated control sample of 12 hours growth,*

339 *Untreated control 24 hours – Untreated control sample of 24 hours growth.*

340 **BamFX1:175 12 hours** BamFX 1:175 sprayed and samples collected after 12 hours ,
 341 **BamFX 1:500 12 hours-** BamFX1:500 sprayed and samples collected after 12 hours ,
 342 **BamFX1:175 24 hours-** BamFX1:175 sprayed and samples collected after 24 hours,
 343 **BamFX1:500 24 hours-** BamFX1:500 sprayed and samples collected after 24 hours.



344

345 **Fig 4.Cumulative effect of fungal infection and Bam-FX treatment**

346 **1:175 24 h-** BamFX1:175 sprayed and samples collected after 24 hours,

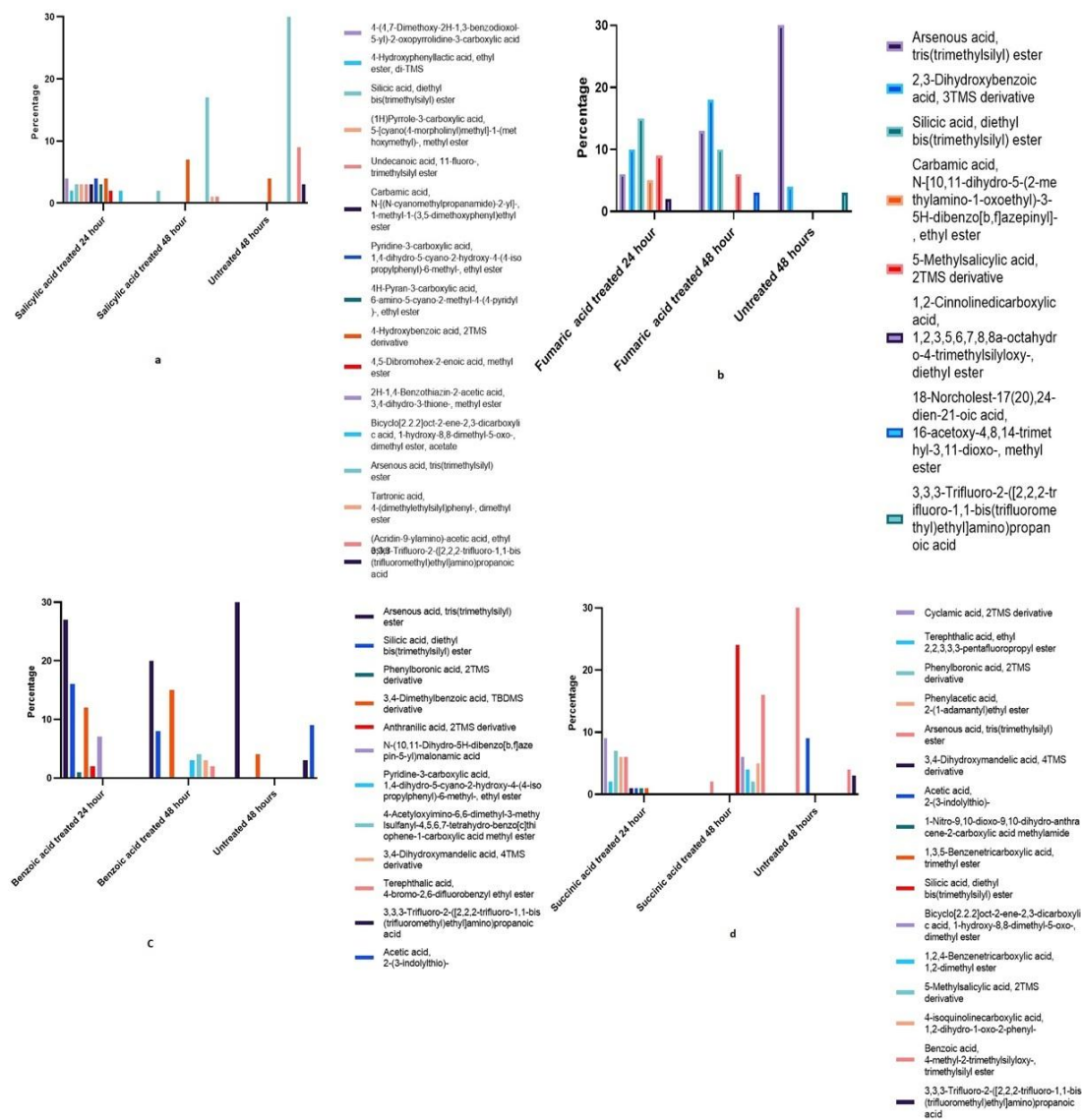
347 **1:500 24 h-** BamFX1:500 sprayed and samples collected after 24 hours,

348 **Positive control of fungal infection** – Aspergillus sp treated Okra seedlings

349 **Untreated control** – Untreated control sample of 24 hours growth.

350

351



352

353 **Fig 5 . Effect of different organic acids on the metabolism of Bam-FX-treated seeds. (a)** Seeds treated

354 with Succinic acid , (b) Seeds treated with Salicylic acid , (c) Seeds treated with Fumaric acid , (d) Seeds

355 treated with Benzoic acid .

356

357

358