

1 ***Symptoms expression of bakanae disease following seed treatment with***
2 ***phytohormone and metabolites***

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20 **Abstract**

21 Gibberellic acid (GA₃) phytohormone responsible for bakanae disease development is
22 well-known and identified. But a number of secondary metabolites along with GA₃, produced by
23 the causal pathogen in relation to bakanae symptoms expression were unknown. Therefore, the
24 aims of this research were to evaluate the symptoms expression analysis of bakanae disease by
25 pre-seed treatment with pure (synthetic) phytohormones and metabolites in susceptible rice
26 variety MR 211. The typical bakanae symptoms were evaluated by applying pure GA₃, FA and
27 MON either singly or in mixtures. It was confirmed that higher concentration of GA₃ singly or
28 with higher concentration of GA₃ and MON in mixtures, caused unusual elongation of
29 internodes. Plants became stunted when high concentration of FA was applied. Browning of
30 leaves and stems, crown rot, root necrosis occurred and root length was decreased when mixtures
31 of higher concentration of FA *MON*GA₃ were used as pre-treatment. Similar observations
32 were noted in plants inoculated with *F. proliferatum* at different score levels. The mechanisms of

33 bakanae disease development through different symptoms expression in susceptible variety
34 infected with *F. proliferatum* were identified.

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36 **Introduction**

37 *Fusarium fujikuroi*, causal agent of bakanae disease is known to produce gibberellic acid
38 (GA₃), fumonisin (FB1), moniliformin (MON), fusaric acid (FA) and beauvericin (BEA) in
39 diseased plant [1- 4]. GA₃ is a growth promoting phytohormone but abnormal production of GA₃
40 has been identified as a break-through in disease development and disease resistance or
41 susceptibility in plants [5]. In addition, GA₃ has been identified as being responsible for increase
42 in plant height, whereas FA is responsible for decrease in plant height. Fungal metabolites MON,
43 FB1 and BEA have been identified for causing phytotoxicity in plants rather than establishment
44 of pathogenicity or disease symptoms expression in bakanae diseased plants [1, 6-7]. Besides
45 *Fusarium fujikuroi*, *Fusarium proliferatum* is also identified as a causal agent of bakanae disease
46 [8] and GA₃, FB1, MON, FA were also isolated from bakanae diseased plants infected with *F.*
47 *proliferatum* [9]. It was also assumed that GA₃, MON and FA had strong influence on bakanae
48 symptoms expression as a significant amounts of GA₃, MON and FA were isolated from the
49 infected susceptible variety (data not presented here). Therefore, this study was carried out to
50 verify the role of GA₃, MON and FA on bakanae symptoms expression, following exogenous
51 pre-treatment on seeds of susceptible variety MR 211 instead of inoculation with *F. proliferatum*
52 over time.

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55 **Materials and methods**

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57 ***Soil mixture***

58 Sand 40%, clay 30% and peat (PEATGRO) 30% were mixed well and sterilized at 120
59 °C for 90 min. Trays (28 cm x 21 cm x 6.5 cm) were filled with 2 kg of the sterilized soil
60 mixture and used for sowing pre-germinated seeds.

61 ***Chemicals used***

62 Pure (Synthetic) GA₃, FA, and MON were used in this study. These chemicals were
63 chosen based on positive relationship that were observed in relation to bakanae symptoms
64 expression in susceptible variety MR 211 (data not presented here). Two concentrations of each
65 chemical (GA₃=15 µg/g, GA₃=10 µg/g, FA= 400 µg/g, FA= 100 µg/g, MON= 120 ng/g, MON= 66 80 ng/g) were used in this study. The pure chemicals GA₃, FA, and MON were purchased from
67 Sigma-Aldrich.

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69 ***Experimental layout and design***

70 Pre-germinated seeds (soaked in water for 48 h) of susceptible variety MR 211 were
71 soaked for 12 h in the probation hormone (GA₃) and metabolite (FA and MON) solutions at two
72 concentrations singly to find out the effective concentration suitable for causing infection or
73 symptoms expression. This moderate pre-treatment period (12 h) was chosen in order to avoid
74 the toxicity caused by the metabolites FA and MON treatment over prolonged periods at higher
75 concentration used and to avoid death of plants at the very early stages of application ^{6, 10, 11}. The
76 two concentrations of each treatment were determined based on the highest and the lowest
77 concentrations derived from bakanae diseased plants (data not presented here). The pre-treated
78 seeds were sown in the sterilized soil in trays. Untreated pre-germinated seeds and pre-
79 germinated seeds inoculated with *F. proliferatum* (10⁶ conidia/mL) used as controls were sown

80 at the same time as phytohormone and metabolite pre-treated seeds. Seeds were sown in two
81 rows per tray with 15 seeds per row. A total of 8 treatments along with 2 controls were used in
82 this experiment. Each tray represented a single replication and trays were arranged in a
83 completely randomized design with 3 replications per treatment (30 seeds per replication). All
84 plants were maintained in a glasshouse with day and night temperatures of 30–35 °C and 23–30
85 °C, respectively, and watered daily. No fertilizer was applied to avoid any effects on the
86 phytohormone and metabolites.

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89 After the single pre-treatment trial, mixtures with two pre-treatment combinations and mixtures
90 with three pre-treatment combinations were employed to observe the symptoms expression in
91 mixtures with different treatment combinations. All procedures used were the same as in the
92 single pre-treatment trial. Development of symptoms associated with the pre-treatments, either
93 singly or in mixtures with two and three pre-treatment combinations was recorded weekly for 3
94 weeks.

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96 ***Sampling procedure and data collection***
97 Plants were uprooted carefully without damaging the root tissues at the different
98 sampling times. Sampling times were determined based on disease score levels as follows: level
99 1 (7 days after pre-treatment), level 3 (14 days after pre-treatment) and level 5 (21 days after
100 pre-treatment). The stems, leaves (uppermost second leaf) and roots were separated, and stem
101 and leaf lengths were measured using a measuring scale (1 m).

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103 Roots were washed with water to remove soil and root length was recorded using the Root
104 Scanner (Winrhizo V700, 2012b). Data expressed as percentage (%) increase or decrease in stem
105 height and root length over the untreated control at each sampling time was analyzed (10 plants
106 per replicate).

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108 ***Effects of pre-treatment with GA₃, FA, and MON on stem cell elongation (Histopathological***
109 ***study)***

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111 Random samples of stem sections from plants pre-treated singly with GA₃ (15 mg/L), FA
112 (400 mg/L), and MON (120 µg/L), in mixtures with GA₃, (15 mg/L) * FA (400 mg/L)* MON
113 (120 µg/L), the untreated control and the diseased control were observed after 14 days of pre-
114 treatment applied. Plants showing typical symptoms of bakanae disease in relation to disease
115 score level 1 and 3 were collected and studied. Stems were cut into 1 cm sections and prepared
116 for scanning electron microscope analysis. Average cell lengths in the longitudinal stem sections
117 of the plants grown from pre-treated seeds and inoculated plants were compared with those in the
118 untreated control plants. Average cell length was measured from 30 randomly selected cells from
119 3 sets of samples (10 cells/sample set) observed under SEM.

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121 ***Analysis of symptoms expression in plants pre-treated with pure GA₃, FA and MON applied***
122 ***singly or in mixtures***

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124 Symptoms expression were analysed in plants pre-treated with pure GA₃, FA, and MON
125 at two concentrations applied singly and in mixtures with two and three treatment combinations
126 at stipulated disease scoring levels (score 1, 3 and 5) on percent increase or decrease basis in
127 comparison with diseased control plants. Data were analysed using SAS software (Version 9.2).

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129 **Results**

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131 ***Effects of GA₃, FA and MON applied singly***

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Both GA₃ concentrations increased stem height, whereas the other pre-treatments decreased stem height after 7 days of pre-treatment. Highest stem height decrease was observed in pre-treatment FA (400 mg/L) after 7 days of pre-treatment. It was also observed that the increasing trend in stem height in GA₃ pre-treatment declined after 7 days to after 21 days. In contrast, pre-treatment with MON (120 µg/L) showed increasing stem height after 14 days of pre-treatment and was found to continue increasing after 21 days which was similar to pre-treatment with GA₃ (15 mg/L). Percent decrease in stem height declined in pre-treatment with FA (400 mg/L) over time. Stem height increase/decrease following the treatment used were observed in Figure 1.

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Figure 1. Stem height increase or decrease in plants following pre-seed treatment with GA₃, FA, and MON singly in comparison with disease free (control) and disease control (inoculated) plants after 14 days.

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These results were further confirmed by the histopathological study comparing cell lengths elongation with the different treatments. Stem cell length (average) was elongated (61.33 µm) in plants pre-treated with higher concentration of GA₃ and was comparable to average stem cell length in diseased control plants (63.47 µm) after 14 days of pre-treatment and inoculation, respectively (Figure 2 a and b). Average cell length of stems was elongated somewhat (47.6 µm) when pre-treated with higher concentration of MON compared to average stem cell length of healthy control plants (45.33 µm) after 14 days (Figure 2 c and d).

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In contrast, pre-treatment with FA showed stunting in stem height, which may be due to the shorter stem cell length. This percentage decrease in stem height observed was probably due to the higher cell division that occurred in plants at disease score 1, which caused shorter cell

157 length (40 μm) in stems as compared to the stem cell length of healthy control plants (45.33 μm)
158 observed in the histopathological study (Figure 2 e and d). From these result, it was evident that
159 higher concentrations of GA₃ and FA were responsible for the stem height increase and decrease,
160 respectively, whereas the higher concentration of MON was responsible for marginal stem height
161 increase after 14 days and onwards.

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163 ***Effects of GA₃, FA and MON applied in combinations***
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165 In mixtures with two treatment combinations, seeds pre-treated with GA₃ (15 mg/L)* MON (120
166 $\mu\text{g/L}$) resulted in the highest (%) increase in stem height after 7 -14 days and was found to the
167 next to the diseased control plants. All combinations with GA₃ and MON increased stem height
168 after 14 days. Therefore, it was apparent that both GA₃ and MON had a synergistic effect on
169 stem height increase. Thus, at the disease score level 3, stem height was increased after 14 days,
170 and this increasing trend was observed in pre-treatment combination treatments with GA₃ (15
171 mg/L)* MON (120 $\mu\text{g/L}$). In contrast, there might be an antagonistic response between FA and
172 GA₃ and stem height increase or decrease was observed in relation to higher concentration of
173 involvement with GA₃ or FA vice versa. Stem height increase in plants of pre-treated seeds with
174 GA₃ (10 mg/L)* FA (400 mg/L) and in inoculated plants (diseased control) were observed to be
175 almost similar after 7days. Thus, stunting observed in plants after 7 days of inoculation might be
176 due to the effect of higher concentration of FA present in plants at the disease score level 1 (after
177 7 days of inoculation).

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179 The mixtures with three pre-treatment combinations of FA (400 mg/L)* MON (80 $\mu\text{g/L}$)* GA₃
180 (15 mg/L) and FA (100 mg/L)* MON (120 $\mu\text{g/L}$)* GA₃ (15 mg/L) increased stem height after 7

181 days. This is largely due to the effect of GA₃ (15 mg/L) singly as the treatment increased stem
182 height early at 7 days after pre-treatment. Although stem cell length was observed to be
183 shortened in this pre-treatment compared to control plants (Figure 2 f and d) but stem height
184 increase after 14 days in this pre-treatment was attributed to the effect of excessive cell divisions
185 in this pre-treatment.

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188 **Figure 2: SEM micrograph of stem cell length in GA₃ (15 mg/L), FA (400 mg/L) and MON**
189 **(120 µg/L) pre-treated plants singly or in combination in comparison with diseased and**
190 **healthy control plants. [GA₃ pre-treated stem cells (a), Stem cells of diseased control plants**
191 **(b), MON pre-treated stem cell (c), Stem cells of healthy control plants (d), FA pre-treated**
192 **stem cell (e) and Stem cells in mixtures of pre-treatment with GA₃ (15 mg/L)* FA (400**
193 **mg/L)* MON (120 µg/L) (f)]**

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195 Along with increases in stem height, leaf browning, stem browning, crown rot and root necrosis
196 were observed in plants pre-treated with MON at both concentrations after 21 days. Browning
197 was found to be more prominent at the lower concentration of MON (80 µg/L) applied singly as
198 pre-treatment, whereas crown rot and root necrosis were more prominent at the higher
199 concentration of MON (120 µg/L) (**Figure 3**). The browning symptoms observed in leaves and
200 stems were similar to the symptoms on plants infected with *F. proliferatum* (diseased control),
201 and could easily be distinguished from the symptoms on untreated control (healthy) plants
202 (**Figure 3**).

203 **Figure 3: Plants pre-treated with MON singly and diseased control plants showing leaf**
204 **browning, stem browning, crown rot and root necrosis in comparison with untreated**
205 **(healthy) control plants after 21 days.**

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207 The highest decrease in root length was observed after 7 days, while it was found to increase
208 after 14 days in all pre-treatments along with the diseased control. The highest decrease in root
209 length was observed in pre-treatment with GA₃ (15 mg/L) followed by the diseased control and

210 GA₃ after 7 days. Both concentrations of MON increased root length after 21 days of pre-
211 treatment but with necrotic lesions.

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213 In mixtures with two pre-treatment combinations the highest root length increase was observed
214 in mixtures with GA₃ (15 mg/L)* MON (120 µg/L) after 14 days (Figure 4a). It was also
215 observed that both concentrations of MON caused crown rot and root necrosis in mixtures with
216 GA₃ in plants pre-treated with two pre-treatment combinations (Figure 5 a). It was also apparent
217 that MON in mixtures either with GA₃ or with FA caused prominent crown rot and root necrosis
218 compared to that in mixtures with FA and GA₃ (Figure 4 b and c).

219

220 **Figure 4: Plant roots showing different architectures when seeds were pre-treated with two**
221 **treatment mixtures. [mixtures with MON and GA₃ (a) and mixtures with FA and GA₃ and**
222 **mixtures with FA and MON (b)]** *G1= GA₃ (10 mg/L), G2= GA₃ (15 mg/L), F1= FA (100 mg/L), F2=

223 FA (400 mg/L), M1= MON (80 µg/L), M2= MON (120 µg/L)]. *MON = moniliformtn, GA₃ = gibberellic

224 acid, FA= fusaric acid

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227 Crown rot and root necrosis was also observed to be severe in mixtures with three pre-treatment
228 combinations in GA₃ (15 mg/L)* MON (120 µg/L)* FA (400 mg/L) (Figure 5).

229

230 **Figure 5. Crown rot and root necrosis due to the effect of MON in mixtures with three pre-**
231 **treatment combinations.** {*G1= GA₃ (10 mg/L), G2= GA₃ (15 mg/L), F1= FA (100 mg/L), F2= FA (400

232 mg/L), M1= MON (80 µg/L), M2= MON (120 µg/L)}. *MON = moniliformtn, GA₃ = gibberellic acid,

233 FA = fusaric acid

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236 From the scenario in pre-treatments, either singly or in mixtures, it was observed that higher
concentrations of GA₃ and MON had a direct influence on increase in stem height. In contrast,

237 FA singly in any concentration or at higher concentrations in mixtures with GA₃ and MON
238 decreased stem height.

239

240 **Discussion**

241

242 Stem height increase in bakanae diseased plants at the disease score level of 3 (14 days
243 after inoculation) was found to be dependent on higher amount of GA₃ singly or in mixtures with
244 MON. Although stem height increase (%) was observed to be lower in pre-treatment GA₃ (15
245 mg/L)* MON (120 µg/L) compared to diseased control plants after 14 days but it was presumed
246 to be due to slow down of GA₃ activity with time. Moreover, increase in GA₃ concentration was
247 initiated in the diseased control plants after 7 days of pathogen inoculation and reached a
248 maximum concentration in infected plants after 14 days, whereas pre-treatment with GA₃
249 responded with a maximum level after 7 days. Similar observations were reported by other
250 researchers, where GA₃ concentration was increased to its highest level in plants after 1 week of
251 application and subsequently decreased with time [12].

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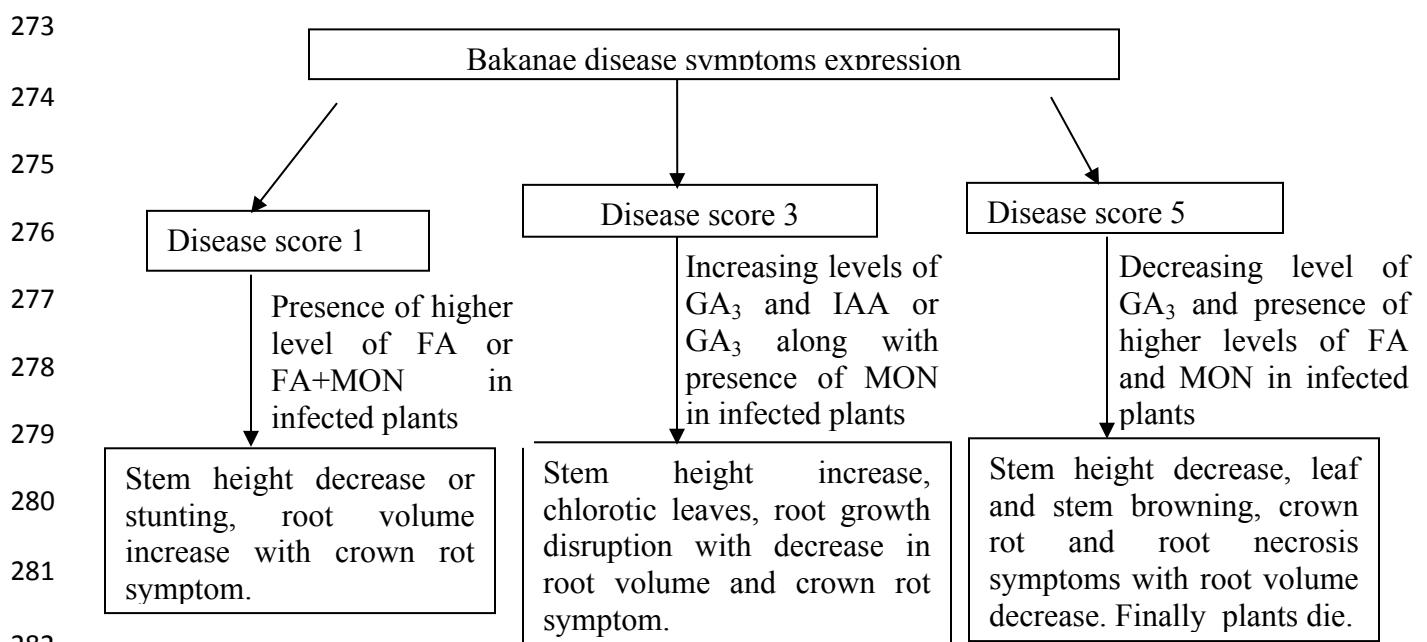
253 In contrast, pre-treatment with higher concentrations of FA singly or in mixtures with MON
254 decreased stem height after 7 days and was reflected in bakanae diseased plants where stunting
255 was observed at the disease score level of 1 after 7 days of inoculation. At the disease score level
256 of 5, stem heights were found to decrease to some extent probably due to the effect of GA₃. A
257 possible explanation for this was that the increasing rate of GA₃ was slowed down and FA was
258 increased significantly due to antagonistic effect with each other with time in infected plants as
259 observed after 21 days of pre-treatment. This is the first report that complex bakanae symptoms

260 associated with the combination effect of increased GA₃ levels along with metabolites FA and
261 MON produced by the pathogen in infected plants, rather than solely dependent on increased
262 levels of GA₃ in infected plants. bakanae symptoms expression due to phytohormonal and
263 metabolites effect is as illustrated in the Figure 6.

264

265 Stem height increase (%) was reliant on elongation of cell length as well as an increase in cell
266 division inside the plant tissues as observed by several researchers. Other researchers reported
267 that cell division was mainly influenced by GA₃ application compared to cell elongation ¹¹. The
268 authors assumed that IAA might have an influence on plant height increase after cell division
269 occurred with GA₃ application. Later, other researchers explained that GA₃ pre-treated plants
270 stimulated IAA synthesis in plant cells first and IAA had an effect on cell elongation and this cell
271 elongation was increased in aged tissues compared to young tissues [14-15].

272



283 **Figure 6: Flowchart of Bakanae symptoms expression due to phytohormonal**

284 **and metabolites effect (GA₃= gibberellic acid, IAA= indole acetic acid, FA=**
285 **fusaric acid, MON= moniliformin).**

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288 Thus, the increase in stem height in bakanae diseased plants was due to the combined effect of
289 cell division and cell length elongation. The mechanism is similar as observed in infected plants
290 with *F. proliferatum* which induced cell division first due to increase levels of GA₃ and thereby
291 stimulated IAA production in infected plants. Thus, IAA in infected plants influenced cell
292 enlargement in bakanae diseased plants. It was also established that IAA levels increased in
293 bakanae diseased plants infected with *F. proliferatum* when GA₃ was increased [9]. Several
294 researchers have also supported that GA₃ and IAA play their role synergistically in plants [15-
295 17]. This higher level of GA₃ along with increased IAA levels at the disease score level of 3,
296 caused stem height increase compared to control plants after 14 days of inoculation. Although
297 some stem height increase and cell enlargement was observed in MON pre-treated stem cells, but
298 this response in relation to IAA increase has not been reported yet.

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300 In the three pre-treatment combination FA (400 mg/L)* MON (120 µg/L)* GA₃ (15 mg/L) stem
301 height was increased (after 14 days) although shorter stem cell length (36.53 µm) was observed
302 compared to the control (45.33 µm) in the histopathological study. The shorter cell length in this
303 three pre-treatments mixture was probably due to an increase in cell division as a result of FA
304 (400 mg/L) and/or GA₃ (15 mg/L) and observed in the histopathological study after 14 days of
305 inoculation. Moreover, it was reported that IAA was increased in plants at the second week after
306 GA₃ application ¹⁰, and that the increased IAA was reflected by a higher increase in stem height
307 after 14 days with pre-treatment FA (400 mg/L)* MON (120 µg/L)* GA₃ (15 mg/L). Again the
308 shorter stem cell length (36.53 µm) was the average of 30 randomly selected stem cells pre-

309 treated with FA (400 mg/L)* MON (120 μ g/L)* GA₃ (15 mg/L). Although some stem cells were
310 initiated to elongate in this pre-treatment as observed in Figure 5.2e whereas, others were in
311 initial stage of elongation and did not reflect individual cell enlargement in histopathological
312 study where measurement was on average cell length. Additionally, stem height increase was
313 slowed down after 7 days due to antagonistic effect of higher concentration of FA with GA₃ and
314 after 14 days stem height increased due to the effect of higher concentration of MON and GA₃
315 combination.

316

317 In contrast, plant height stunting was found to be mainly due to the effect of FA rather than GA₃.
318 This observation is supported by other researchers as well. Other researcher reported that plant
319 stunting or rosetting occurred due to low concentration of GA₃ in plants ¹⁶. It was also observed
320 that high levels of FA accumulation in plants resulted in stunted plant height and decreased **root**
321 length in tomato [7, 19-20]. The lower amount of GA₃ (8.9 μ g/g fresh wt.) as determined in the
322 disease score level of 1 resulted in stunting of plants after 7 days of pre-treatment. Although
323 metabolic activity was not determined in this experiment but other researchers reported that the
324 stunting mechanism is mainly due to changes in metabolic activity in plants, including speeding
325 up of the lipid peroxide activity, inhibition of ATP synthesis or decreased ATP levels [6, 19-20].
326 compared to control plants. Moreover, higher concentrations of FA and MON also resulted in
327 plant height reduction as was observed in jimsonweed plants when pure FA and MON were
328 applied ⁷. Thus, lower amount of GA₃ and/or in mixtures with higher amounts of FA and MON
329 contributed to the cessation and collapse of plants growth at the disease score level of 5 that was
330 observed after 21 days of pre-treatment.

331

332 Crown rot and root necrosis due to higher concentration of MON in infected plants at later
333 growth stages were observed. Similar symptoms have also been observed by other researchers in
334 different plant species [7, 21]. Brown to pink discoloration of leaves at a disease score level of 5
335 may be due to higher concentration of MON present in plants infected by *Fusarium*
336 *proliferatum*. It was also observed pink ear rot in maize when a higher concentration of MON
337 was associated with the ear rot causal pathogen *Fusarium suubgltinans* [22]. In addition, earlier
338 researcher reported that MON had toxic effects with growth reductions of coleoptiles as well as
339 vein chlorosis and necrosis in corn and tobacco callus ¹⁹. MON was also found to cause
340 cytoplasmic disruptions and abnormal mitosis that resulted in “a disruption of the spindle
341 apparatus” in infected plant cells ^{19, 21}. In contrast, reported that browning of stalk, leaf, and ear
342 of rice was due to the effect of fumonisin B1 when inoculated with *F. proliferatum* ²². However,
343 the authors did not isolate other mycotoxins produced by the fungus in the rice plants infected
344 with *F. proliferatum* nor compared the symptoms treated with pure fumonisin B1. More recently
345 provided evidence that FB1 had no pathogenicity effects on bakanae symptoms development ¹.
346 The browning or pinkish symptom associated with a disease score 5 was evaluated by applying
347 pure MON to germinating seeds of susceptible variety MR 211, and similar symptoms were
348 observed as in plants inoculated with *F. proliferatum*. Therefore, it was confirmed that browning
349 and pinkish discoloration of leaves and stems as well as crown rot and root necrosis were
350 associated with MON effect. Higher concentration of FA, MON and less GA₃ accumulation in
351 infected plants resulted in dead plants over time at a disease score level of 5, and this was
352 attributed to inhibition of photosynthesis and toxic effects of MON and FA in plant cells.
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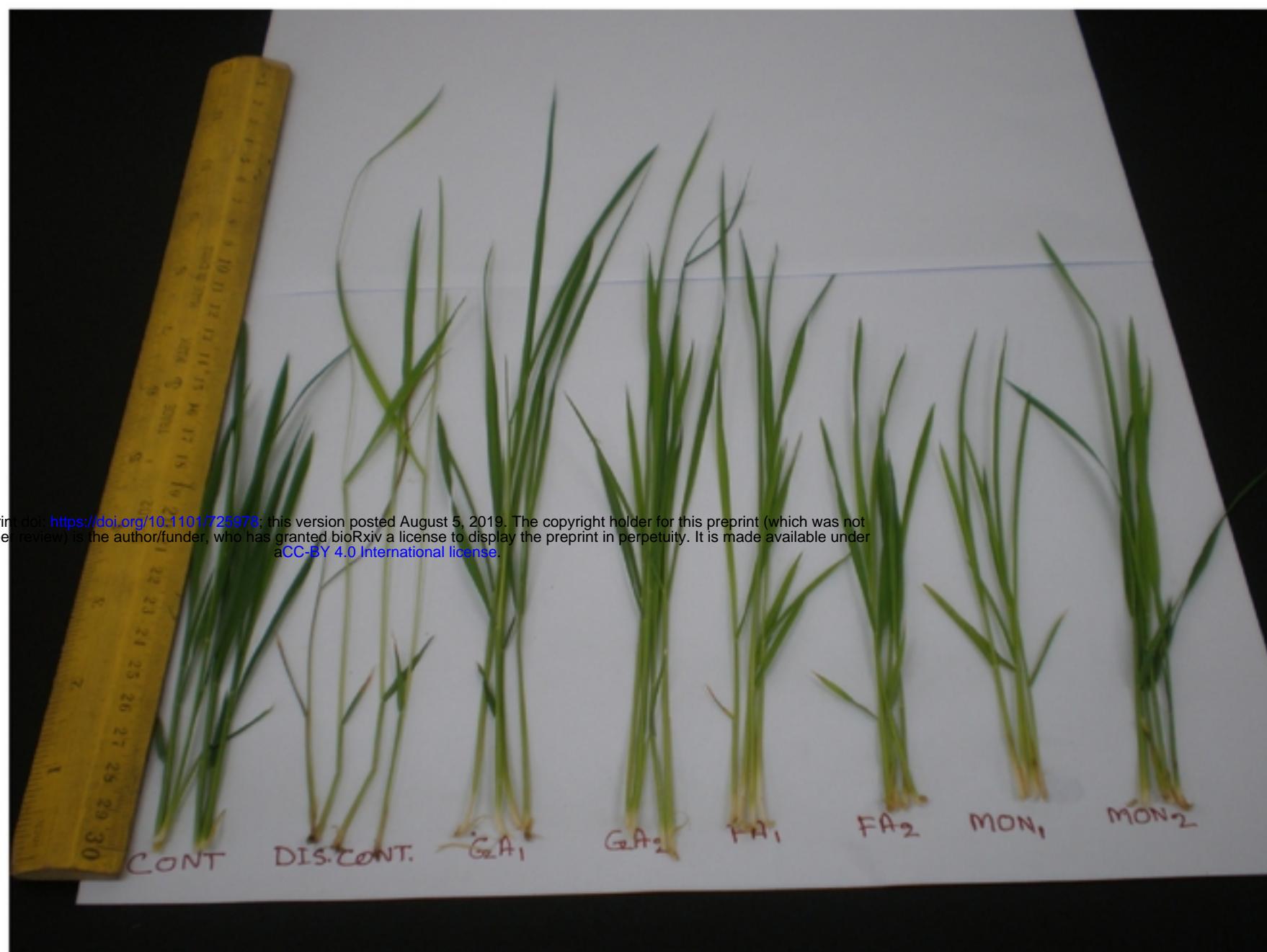


Figure 1. Stem height increase or decrease in plants following pre-seed treatment with GA₃, FA, and MON singly in comparison with disease free (control) and disease control (inoculated) plants after 14 days.

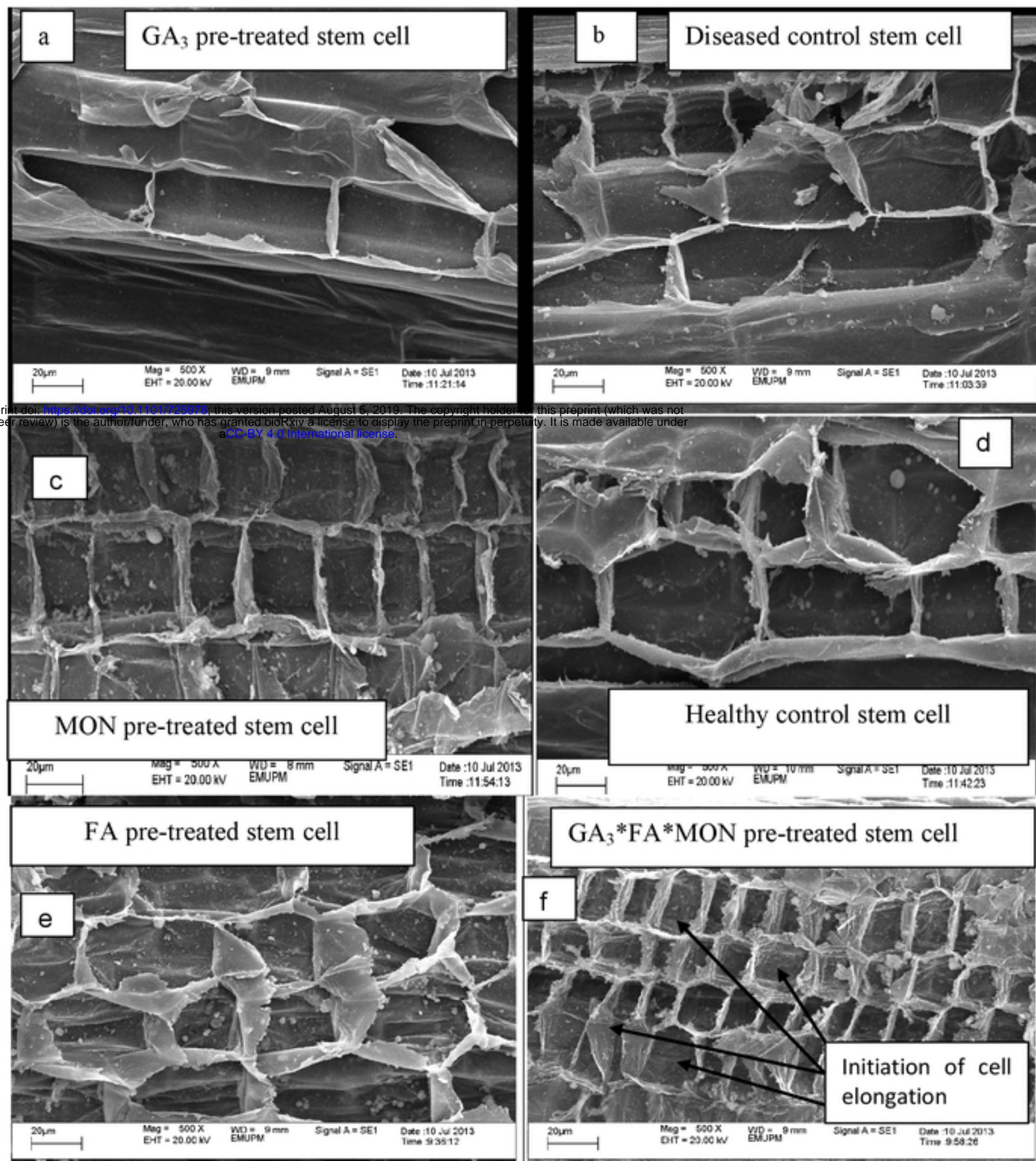
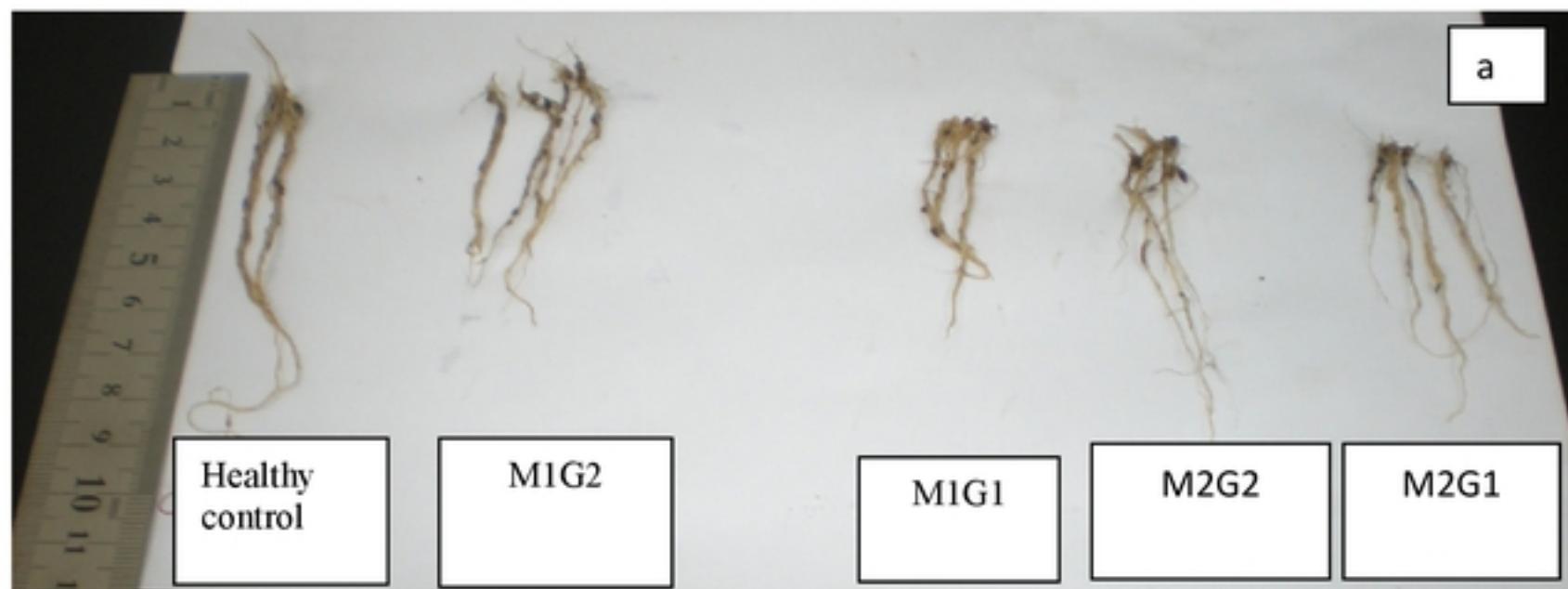


Figure 2: SEM micrograph of stem cell length in GA₃ (15 mg/L), FA (400 mg/L) and MON (120 µg/L) pre-treated plants singly or in combination in comparison with diseased and healthy control plants. [GA₃ pre-treated stem cells (a), Stem cells of diseased control plants (b), MON pre-treated stem cell (c), Stem cells of healthy control plants (d), FA pre-treated stem cell (e) and Stem cells in mixtures of pre-treatment with GA₃ (15 mg/L)* FA (400 mg/L)* MON (120 µg/L) (f)]

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Figure 3: Plants pre-treated with MON singly and diseased control plants showing leaf browning, stem browning, crown rot and root necrosis in comparison with untreated (healthy) control plants after 21 days.



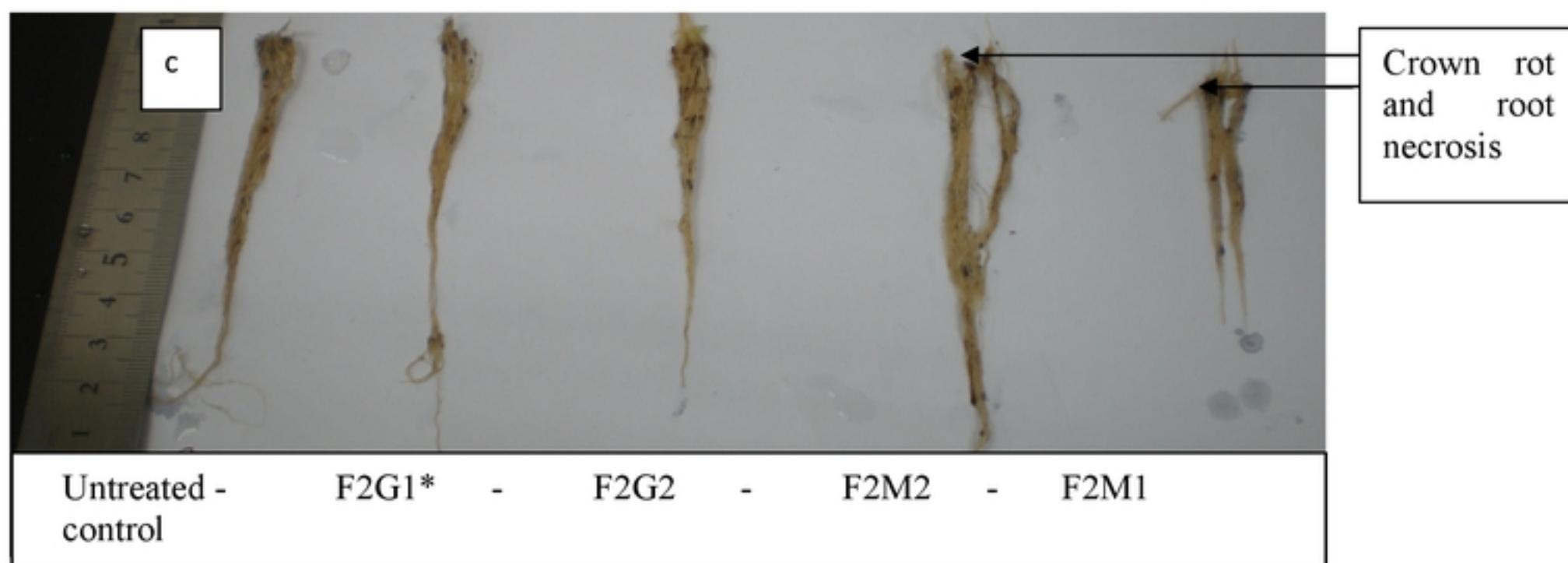


Figure 4: Plant roots showing different architectures when seeds were pre-treated with two treatment mixtures. [mixtures with MON and GA₃ (a) and mixtures with FA and GA₃ and mixtures with FA and MON (b)] *G1= GA₃ (10 mg/L), G2= GA₃ (15 mg/L), F1= FA (100 mg/L), F2= FA (400 mg/L), M1= MON (80 µg/L), M2= MON (120 µg/L)]. *MON = moniliformtn, GA₃ = gibberellic acid, FA= fusaric acid

combinations in GA₃ (15 mg/L)* MON (120 µg/L)* FA (400 mg/L) (Figure 5).



Figure 5. Crown rot and root necrosis due to the effect of MON in mixtures with three pre-treatment combinations. {^{*}G1= GA₃ (10 mg/L), G2= GA₃ (15 mg/L), F1= FA (100 mg/L), F2= FA (400 mg/L), M1= MON (80 µg/L), M2= MON (120 µg/L)}. *MON = moniliformtn, GA₃ = gibberellic acid, FA = fusaric acid