

2 **Priming Induction in Neighbouring Plants of**
3 ***Gossypium hirsutum* under Salt Stress**

4
5 **ABSTRACT**

6 Plants are subjected to various types of environmental stresses throughout their lifecycle. It has been
7 found that plants are able to communicate with the neighbouring plants under stress conditions
8 through volatile organic compounds. These volatiles act as signals for the neighbouring plants thus
9 preparing them for the upcoming stress, a phenomenon known as priming. So, the present study
10 explores the effects of salt stress on cotton plants and the resultant induction of priming in the nearby
11 plants. For this purpose, salt tolerant cotton (*Gossypium hirsutum*) variety was used. Two
12 concentration levels, 100 mM, and 150 mM (what) were used to study the impacts of the stress. The
13 experiment was divided into two steps for each treatment. In the first step, a set of plants (emitters)
14 was given salt stress. The second set of plants (receivers) was placed adjacent to the stressed plants
15 (emitters), while the third set of plants was placed separately as a control for both the treatments.
16 Various physiological and morphological parameters were measured at the beginning and the end of
17 the first step. In the second step, the receiver plants now termed as "primed" were given the same
18 levels of stress while a new set of non-primed plants was placed near the primed plants. These non-
19 primed plants were now treated with 100mM and 150mM of NaCl respectively and the results were
20 compared. The results show that plants were able to get signals from neighbouring stressed plants.
21 Plants responded by altering morphology and physiology to prepare themselves for future stress
22 conditions.

23 **1. INTRODUCTION**

24 Communication among plants has been an area of interest for quite a long time now. This
25 communication is done by releasing and sensing volatile organic compounds. These compounds are
26 released by the plants under normal circumstances [1] and are found in almost all plant tissues
27 including roots, stem, leaves and flower tissues.

28 However, under conditions of stress, the level and composition of these volatile organic compounds
29 have been reported to fluctuate [2]. Several studies suggest that plants are also able to receive
30 signals from neighbouring plants in the form of these VOCs. Plants can thus detect any change in the
31 concentration of such signals, from a neighbouring plant, caused by biotic or abiotic stress [3, 5].
32 Interaction among plants through roots via common mycorrhizal networks (CMNs) has already been
33 reported in several studies

34 The present study focuses on the plant to plant communication as a result of VOC emission through
35 the leaves. VOCs released by leaf tissues are called "Green Leaf Volatiles" or GLVs [6, 7]. Alterations
36 in the levels of GLVs can induce defensive responses in the form of physiological and morphological
37 changes in the unstressed neighbours [8]. Green leaf volatiles mainly consists of alcohols,
38 aldehydes, ketones, monoterpenes and esters [9]. Several studies show the effects of biotic stresses
39 on GLVs concentration such as viral infection [10, 12].

40 However, there is not much work done over the effects of abiotic stress on the levels of VOCs and the
41 resultant induction of resistance (priming) in the receiver plants. Salinity is abiotic stress and is
42 associated with several changes in the treated plants at both morphological as well as physiological
43 level. Changes in the levels of isoprene and other volatile organic compounds have been associated
44 with salinity stress. So the following experiment was conducted in order to study the effects of salinity
45 stress on both treated and untreated cotton plants as well as the induction of priming in the untreated
46 neighbouring plants.

2. MATERIAL AND METHODS

The experiment was carried out at Cotton Research Station, Ayub Agricultural Research Institute Faisalabad. Fuzzy seeds (age) of cotton (*Gossypium hirsutum*) variety FH-142 were soaked under water overnight. These seeds were then sown in polythene bags containing a mixture of compost and alluvial soil (a mixture of sand silt and clay) for good germination. 210 bags were filled and 4-5 seeds (uniform treatment) were sown in each bag on 02 March, 2018 and placed it in green house.

Transplantation was done after three weeks from polythene bags to plastic pots having one feet height. These pots were filled with soil, alluvial soil and FYM mixture in 4:4:1 proportion. Selected 150 pots and place them under the tunnel and watered it frequently.

The experiment was arranged into three plots for treatment 1, 2 and control respectively and these plots were partitioned by polythene paper. Total 36 plants were placed in 1st and 2nd plot each, while the third plot contained 72 plants. In 1st and 2nd plot, arrange the plants in three rows and each row containing 12 plants. The mid row called emitter while other two rows called receiver, we named it because according to the hypothesis, emitter release volatile organic compounds when we gave any stress while receiver which is placed at the periphery of emitter receive those compounds (Fig. 1). After two weeks of transplantation, salt stress was given to emitters only. Two levels of NaCl concentration were used, 100mM and 150mM respectively. The emitters placed in the 1st plot was given 100 mM solution while other emitters placed in 2nd plot was given 150 mM solution while rest of the plants were on tap water. 100 ml salt solution to emitters and 100 ml tap water to rest of the plants were given.

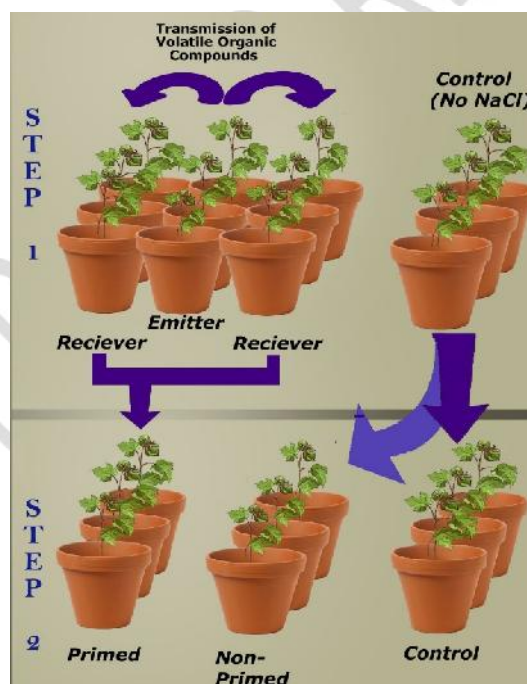


Fig.1 shows the mechanism of the experiment

Since the whole experiment was carried out in two steps. In step one a group of plants called receivers placed in the proximity of salt-stressed plants called emitter for twenty days. Another group of plant called control placed apart from emitter and receiver. In second step, receiver plants were taken from step one (Fig. 1). Now it is called primed plants, gave salt stress to primed and non-primed plants (taken from the control of step 1) and compare it with control. This whole experiment was repeated under different dose of salt concentration.

The parameters that studied are Leaf area, Total dry weight, Total Fresh weight, Relative growth rate, Proline content, No. of leaves per plant, Shoot length, Root length, Relative growth rate, H₂O₂ species, Dry shoot weight, Dry root weight, Moisture content, Root to shoot ratio, Leaf Area.

The plant material with a sample size of six was taken at the beginning of the experiment (day 0), at the end of step 1 just after 20 days of the experiment started and at the end of step 2, after 20 days of step 1 finished. Plants were uprooted carefully, gently washing away the soil and take the fresh weight of each sample by using weighing balance. A different morphological trait that is mentioned above was then analysed. Leaf area was measured using leaf area meter. Plant material was then oven dried at 70 °C for 48 hours. The relative Growth rate was measured by using total dry weight according to Pérez [13] method using the formula $RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1)$, where W is a total dry weight and t is a time between two reference period which is 20 days.

Proline content was determined by Bates [14] method with little modification. Take 250 g sample of ground leaf and add 10ml of 3% solution of salphosalicyclic acid, then centrifuged it under 3000rpm for 10 min, transfer 2ml of supernatant to new test tube after centrifugation. Add 2ml of 6M ortho phosphoric acid, 2ml acid ninhydrin and 2ml glacial acetic acid. Keep it in the water bath for one hour at 100°C. After it adds 4ml of toluene, shake it well and wait for a few minutes. Separate the upper layer of solution and observe it in spectrophotometer under 520nm. The reading was compared with standards. H₂O₂ analysis was determined by Orozco-Cardenas and Ryan [15] method.

2.1 STATISTICAL ANALYSIS

LSD test was used to compare the variation within and between the two treatments for each parameter, while paired t-test was used to compare the step 1 with step 2. This statistical analysis was conducted using the software Statistix 8.1. One-way ANOVA, Mean comparison test and Student's t-test, depending on the dataset, were used to identify significant differences between and within the treatments (P≤0.05).

Table no. 1 shows the variation within and between treatments of emitter and receiver plants in step 1 of the experiment

Step 1		Control	Treatment 1		Treatment 2	
			Emitter	Receiver	Emitter	Receiver
Physiological Data	Proline Content (520nm) $\mu\text{mol g}^{-1}$	0.078 ^C	0.088 ^B	0.091 ^B	0.079 ^C	0.102 ^A
	H ₂ O ₂ Species (390nm)	0.392 ^D	0.499 ^B	0.363 ^D	0.574 ^A	0.471 ^C
	Moisture Content	15.042 ^A	9.091 ^B	9.629 ^B	6.835 ^C	6.566 ^C
Morphological Data	Relative Growth Rate (g)	0.170 ^A	0.131 ^B	0.137 ^B	0.107 ^C	0.107 ^C
	Total Fresh Weight (g)	19.218 ^A	12.498 ^B	12.418 ^B	9.756 ^C	9.498 ^C
	Total Dry Weight (g)	4.177 ^A	3.407 ^B	3.517 ^B	2.922 ^C	2.932 ^C
	Dry Shoot Weight (g)	3.263 ^A	2.818 ^{AB}	2.79 ^{AB}	2.414 ^{BC}	2.245 ^C
	Dry Root Weight (g)	0.913 ^A	0.588 ^C	0.720 ^B	0.507 ^D	0.687 ^B
	Root Length (cm)	14.200 ^A	12.420 ^C	12.440 ^C	12.867 ^{BC}	13.250 ^B
	Shoot Length (cm)	23.125 ^A	21.867 ^B	21.333 ^{BC}	20.967 ^C	20.683 ^C
	Root / Shoot in Weight (g)	0.280 ^A	0.209 ^B	0.257 ^{AB}	0.210 ^B	0.306 ^A
	Root / Shoot in Length (cm)	0.614 ^{AB}	0.568 ^C	0.583 ^{BC}	0.614 ^{AB}	0.641 ^A
	No. of Flowers / 6 plants	13	7	5	6	4

	No. of Leaves	9 ^A	8 ^A	8 ^{AB}	8 ^{AB}	7 ^B
	No. of Nodes	7 ^A	6 ^{AB}	6 ^{AB}	6 ^B	6 ^B

102 Table No. 2 shows the variation within and between treatments of primed (receiver
103 plants were taken from the first step of experiment) and non-primed (control plants
104 were taken from first step of experiment) plants in step 2 of the experiment

Step 2		Control	Treatment 1		Treatment 2	
			Primed	Non-Primed	Primed	Non-Primed
Physiological Data	Proline Content (520nm)	0.205 ^B	0.183 ^C	0.139 ^D	0.209 ^B	0.294 ^A
	H ₂ O ₂ Species (390nm)	1.029 ^B	0.777 ^C	0.569 ^D	1.096 ^B	1.45 ^A
	Moisture Content	13.514 ^A	10.206 ^C	12.421 ^B	6.951 ^E	8.297 ^D
Morphological Data	Relative Growth Rate (g)	0.061 ^{BC}	0.088 ^A	0.065 ^B	0.045 ^C	0.011 ^D
	Total Fresh Weight (g)	18.916 ^A	14.754 ^C	17.907 ^B	10.782 ^E	12.701 ^D
	Total Dry Weight (g)	5.403 ^A	4.548 ^B	5.485 ^A	3.832 ^C	4.404 ^B
	Dry Shoot Weight (g)	4.154 ^B	3.385 ^B	4.235 ^A	2.816 ^C	3.266 ^{BC}
	Dry Root Weight (g)	1.249 ^A	1.163 ^{AB}	1.250 ^A	1.015 ^B	1.138 ^{AB}
	Root Length (cm)	23.400 ^A	21.650 ^B	14.300 ^D	18.833 ^C	14.260 ^D
	Shoot Length (cm)	23.683 ^B	22.750 ^C	24.800 ^A	20.967 ^D	23.220 ^{BC}
	Root / Shoot in Weight (g)	0.301 ^A	0.343 ^A	0.295 ^A	0.360 ^A	0.348 ^A
	Root / Shoot in Length (cm)	0.988 ^A	0.952 ^A	0.577 ^C	0.898 ^B	0.614 ^C
	No. of Flowers / 6 plants	13	8	11	5	7
	No. of Balls / 6 Plants	2	6	3	4	1
	No. of Leaves	9 ^{AB}	8 ^{AB}	10 ^A	7 ^D	7 ^{CD}
No. of Nodes	8 ^{AB}	7 ^{AB}	9 ^A	7 ^B	7 ^{AB}	

105 Table No. 3 shows the data of Change in reading in % from step 1 to step 2

Change in reading in % from step 1 to step 2		Control	Treatment 1		Treatment 2	
			Primed	Non-Primed	Primed	Non-Primed
Physiological Data	Change in Proline Content (520nm)	162.82*	101*	78*	104*	276*
	Change in H2O2 Species (390nm)	162.5*	114*	45.1*	132.7*	269.9*
	Change in Moisture Content	-10.15 N.S	5.99 N.S	-17.42 N.S	5.86 N.S	-44.84 *
Morphological Data	Change in Total Fresh Weight (g)	-1.59 N.S	18.81 N.S	-7.32 N.S	13.51 N.S	-51.31 *
	Change in Total Dry Weight (g)	29.35 *	29.31 *	31.31 N.S	30.69 *	5.43 N.S
	Change in Dry Shoot Weight (g)	27.3 *	21.32 *	29.78 N.S	25.43 N.S	0.09 N.S
	Change in Dry Root Weight (g)	36.8 *	61.52 *	36.91 N.S	47.74 *	24.64 N.S

	Change in Root Length (cm)	64.79 *	74.03 *	0.7 N.S	42.13 *	0.42 N.S
	Change in Shoot Length (cm)	2.41 N.S	6.64*	7.24 *	1.37 N.S	0.41 N.S

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107

108 3. RESULTS

109 3.1 PROLINE CONTENT

110 The experiment was organized into three treatments with two doses of NaCl concentration and one is
 111 controlling. As we see in Table 1, the emitter and receiver of treatment 1 are non-significant while
 112 both are significant with control. It means that the Emitter which is under salt stress produce more
 113 proline than control and it induces a receiver plant to produce proline. When we move this receiver to
 114 step 2 as shown in fig. 3, it becomes primed and shows a significant result with control as well as
 115 Non-primed but it seems that when plant grow the rate of proline content in primed plant reduces and
 116 it produces less amount of proline than control while non primed plant also reduces its rate as
 117 compared with control and receiver.

118 In treatment 2 (under 150 mM NaCl), emitter shows non-significant with control. The treatment 2 has
 119 no effect on proline while it induces proline in receiver. It is assumed that emitter plant produce
 120 another compound and reduces the amount of proline and that another compound induces a proline
 121 in the receiver. When we go to step 2, a receiver gives a better result than treatment one, its proline
 122 content increase up to 104% and becomes equal to control. Non primed plants went to more stress
 123 and it proline content rises up to 276%.

124 3.2 H₂O₂ SPECIES

125 At the end of step one, the emitter produces more H₂O₂ than control and have no effect on receiver
 126 under treatment 1. When this receiver moves to step 2, it shows a negative response compared with
 127 control. The non-primed plant coming from the control of step 1 also shows a negative response.
 128 Overall treatment 1 do not induce a priming effect on neighbouring plants in case of H₂O₂ species.

129 In treatment 2 emitter produces large amount of H₂O₂ and induces in the neighbouring plant. Receiver
 130 shows a priming effect in step 2 and increases its production up to 132.7% and tried to near control.
 131 Non-primed plants increase H₂O₂ up to 269.9%.

132 3.3 MOISTURE CONTENT

133 At the end of step 1 fig. 2, salt greatly effects on the emitter and this emitter induce a priming effect on
 134 the receiver. Moisture content decreases compared with control. In step 2 receiver and control
 135 undergoes no changes while non-primed plant coming from the control of step 1 shows a negative
 136 response. Non-primed plant reduces its moisture content in treatment 1 and 2 up to -17.42% and -
 137 44.84 respectively.

138 3.4 RELATIVE GROWTH RATE

139 Relative growth rate means, how much increase in total dry weight per day. The emitter and receiver
 140 show non-significant results with each other while both show a significant result with control. This
 141 result shows that the emitter and receiver reduce its growth rate in both treatments but if we compare
 142 treatments with each other, it shows that the treatment 2 reduces more growth rate with treatment 1.

143 In step 2 receiver of both treatments show a non-significant result with control. This shows that
 144 receiver which is now primed speed up its growth rate and becomes equal to control. Non-primed
 145 plant in treatment 1 shows non-significant with control; this is due to the stagnant growth of plants in

step 2. Non-primed in treatment 2 greatly reduces its growth rate due to the high level of NaCl concentration.

3.5 PLANT WEIGHT

Plant weight is a major character to study salt stress in plants. Total fresh and dry weight of emitter and receiver in both treatments of step 1 shows a significant reduction in weight. The fresh weight decrease in emitter as well as a receiver might be due to a decrease in moisture content and dry root weight. In step 2 total fresh weight of receiver shows a non-significant result due to no change in moisture content.

Under treatment 2, totally fresh and dry weight of emitter and receiver both show the non-significant result with each other, which shows that the emitter induces a priming effect on the receiver. In step 2 the increase in fresh weight was non-significant because receiver after induction start to increase the total dry weight of shoot, while moisture content remains stagnant.

3.6 PLANT HEIGHT

At the end of step 1, both root and shoot length increases up to definite length but emitter and receiver of both treatments show a significant reduction in length of both character compared with control. Root length of primed in step 2 of treatment 1 and 2 increases up to 74.03% and 42.13% respectively. While the non-primed plant shows no increase in root length compared with control. Shoot length in step 2 show non-significant due to stagnant growth.

3.7 NUMBER OF FLOWERS AND BOLLS

A number of flowers per six plants was counted at the end of step 1. Controls showed the maximum number of flowers per six plants. The number of flowers was significantly reduced in the emitters and receivers in both the treatments.

The no. of flowers per six plants remained the same for control plants. However, the no. of flowers in treatment 1 primed plants increased from 5 flowers to 8 flowers. No. of flowers in the non-primed plants of treatment 1 decreased from 13 to 11. In treatment 2, no. of flowers in the primed plants increased from 4 to 5 while their no. reduced from 13 to 7.

At the end of step 2, an average of two bolls appeared on the control plants. 6 bolls appeared on the primed while 3 appeared on the non-primed plants of treatment 1. Similarly, 4 bolls were formed on the primed while 1 boll was appeared on average in the non-primed plants of treatment 2.



Fig. 2 shows step 1 of experiment Fig. 3 shows step 2 of the experiment

4. DISCUSSION

180 Priming is a phenomenon whereby plants once exposed to a particular stress, become tolerant
181 towards it as a result of physiological and morphological changes. Several studies have been
182 conducted to study plant-to-plant communications caused by biotic and abiotic stresses [16]

183 This experiment was conducted to study whether the neighbouring cotton plants were able to receive
184 airborne signals from the salinity stressed plants. As indicated by the table, although the receivers
185 were not directly exposed to the salt stress, their dry and fresh weights were still reduced compared to
186 the controls. Salinity has been associated with lowering of both fresh and dry weights and it is
187 inversely proportional to the salt concentration [17, 19].

188 The reduction in relative growth rate of the receivers compared with the control indicates the
189 possibility of some sort of airborne communication between the emitters and the receivers.

190 As indicated (Table 1), the fresh and dry weights of the cotton emitter and receiver plants affected by
191 150mmol salt concentration were much lower than those affected with 100mmol salt concentration.
192 Similar effects were observed in the case of dry root weight. Salt stress has been found to be
193 associated with a reduction in total moisture content of the plant [20, 21].

194 Same was the case with the emitters in both the treatments. Interestingly, the moisture content of the
195 neighbouring plants was also lowered indicating the possibility of the plant to plant communication.
196 Salinity causes a reduction in root and shoots length leading to stunted growth [22, 25].

197 By the end of step 1, flowering had been induced in the cotton plants. The number of flowers in the
198 emitter and receiver plants of both the treatments was much less than the control plants.

199 **4.1 STEP 2**

200 Step 2 was conducted to check whether the receivers in step 1 had developed resistance against the
201 same stress they were exposed to as receivers.

202 The data for various morphological and physiological parameters were collected after a 20 days
203 interval.

204 Therefore, 100mmol treatment had a little effect on the non-primed and primed plants. Whereas, the
205 150mmol treatment caused a significant decline in the moisture content of non-primed plants. No
206 significant effect was observed on the primed plants indicating that the plants had developed
207 resistance against the stress.

208 Similar to the moisture content, no significant change was observed in primed and non-primed plants
209 in treatment 1 while the moisture content of non-primed plants was significantly lower than the primed
210 plants. This can be attributed to the loss in moisture content due to salinity stress.

211 There was no increase in the total dry weight of non-primed plants in both the treatments. While the
212 dry weight increased for control and the primed plants, it remained stagnant in the non-primed plants
213 indicating stagnant growth. Root length is reduced by salt stress. Though the root length increased
214 normally in the control and the primed plants, there was no significant increase in the root length of
215 the non-primed plants in both the treatments. Low levels of salinity have positive effects on shoot
216 length at a certain time of development.

217 When the treatment 1 primed and non-primed plants were treated with 100mmol salinity, their shoot
218 length increased. There was no significant increase in the short length of treatment 2 primed and non-
219 primed plants, however, in comparison with the non-primed plants, the shoot length increased more in
220 the primed plants. Flowering is inversely affected by salinity.

221 As indicated by the graphs, the flowering was greatly reduced in treatment 2 non primed plants in
222 comparison to the controlled. Salinity induces boll formation in the cotton plants. Rapid boll formation
223 in the primed plants in both treatments indicates acclimatization to salinity.

224 **4.2 CHANGES IN PHYSIOLOGY**

In step 1, the proline content of both the emitters and the receivers increased slightly at 100mmol concentration. At 150mmol concentration, there was no increase in the proline content but the proline content of the receivers was much higher [26].

This is probably due to the formation of some other compound at higher salt concentration [28] Salinity causes increase in the H₂O₂ species [27].

As indicated, the H₂O₂ species increased at both treatment levels. Interestingly, the H₂O₂ concentration also increased in the receivers indicating communication.

In step 2, the proline content of treatment 2 non primed plants was more than the primed plants indicating preparedness. However, the proline content of control also increased which was due to the growth of the plant [28]. Same was true for H₂O₂ species.

5. CONCLUSION

From our study it is concluded that cotton plants were able to perceive stress signals emitted from stressed neighbours and responded by changing physiology and morphology, possibly preparing them for future stress. The effects were more prominent at 150mM concentration. The result showed that the plants might have communicated with each other through airborne signals and priming was induced on reception of those signals. It is assumed that the tolerance of cotton plants against abiotic stresses may be increased by artificially applying volatile organic compounds.

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