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Effective appraisement of hormones and *Acinetobacter* on salt-stress induced *Cicer arietinum* Cultivars

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Abstract
An experiment conducted to evaluate the effect of hormones and *Acinetobacter* in salt-stressed induced Chickpea cultivars. In this experiment, salinity levels imposed were 4dS/m, 8dS/m and 12dS/m were tested by using conductivity meter. The stock of growth hormones was prepared as 5mg/L and 3 days incubated bacterial suspension culture (*Acinetobacter spp.*) was used for further study. This experiment shows that the germination percentage, seed vigor index, root, and shoot length significantly decreases with an increase in salt concentration. In the analysis of biochemical level, as the increases salt stress, there was a decrease in chlorophyll and protein content but increased in Catalase, Superoxide dismutase and proline were increased. After the treatments of *Acinetobacter* and growth hormones, there were different results observed in morphological and biochemical parameters. Salt stressed induced plants treated with *Acinetobacter* and growth hormones were showed high root-shoot length and increased in chlorophyll and protein content and decreased in Catalase, Superoxide dismutase, proline content. In SDS-PAGE analysis, both cultivars showing extra bands in increased salinity stress but after the treatment of *Acinetobacter* and growth hormones, these bands were disappeared. On the above result, *Acinetobacter* and growth hormones were responsible for maintaining the catalase, SOD, chlorophyll, proline and protein content and also responsible for regaining the root-shoot length. Hence, *Acinetobacter* and growth hormones treated plants show a better result to overcome problems that occurred with salt stress. Growth hormones showed a better result than *Acinetobacter*, Specially NAA worked more prominently than IBA. NAA shows better results as compared to IBA on chickpea and nullifies the effect of salt stress and overcome the problem occurred with salt stress.

Keywords: *Acinetobacter, Cicer arietinum,* growth hormones, salt stress

1. Introduction

Chickpea (*Cicer arietinum* L.) is a legume crop and belongs to the family Fabaceae, kingdom Plantae, division Magnoliophyta, class Magnoliopsida, order Fabales, family Fabaceae, subfamily faboideae, genus Cicer and species *Cicer arietinum* [27]. Chickpea is self-pollinated, diploid (2n=2x=16) with a genome size of 740 Mbp. It is an annual crop that can complete its life cycle in 90 to 180 days depending on the prevailing meteorological conditions [10]. Chickpea is the third most important pulse crop in the world in terms of total production which is mostly grown in semi-arid regions such as South Asia, West Asia, North Africa, East Africa, Southern Europe, North and South America, and Australia. It is cultivated in more than 50 countries with over 11 million hectares, and its total annual world production is around 8.4 million tons [1]. Chickpea is a valuable source of protein, carbohydrate, fibre and many essential vitamins and minerals. Chickpea nitrogen fixation plays an important role in maintenance of the soil fertility, particularly in the arid and low rainfall areas [4]. In cereal-based diets, chickpea seed is a protein-rich source [26]. Chickpea is one of the oldest crops grown mainly for their seed which contain 20.6 % protein, 2.2% fat and 61.2% carbohydrates [19]. Stress in plants can be defined as any external factor that negatively influences plant growth, productivity, reproductive capacity or survival [28]. The crop growth and development are constantly influenced by environmental conditions such as stresses which are the most important yield reducing factors in the world [15]. Salt stress that occurred in the soils with high salt reduces the ability of plants to absorb water, and this quickly reduces seedling growth [17]. Salinity is one of the major obstacles in increasing production in chickpea growing areas. Salinity stress is a major environmental factor that extremely affects crop production throughout the world, it is a danger to both agriculture and the soil body [19]. It is estimated that around 20% of total land in the world and nearly half of all irrigated land are adversely influenced by salinity stress. Salinity causes not only physiological dehydration (water stress) in plants, but nutrient ion imbalance. Under saline conditions, reactive oxygen species (ROS) are commonly generated and accumulated by which oxidative damage occurs in bio-molecules such as lipids and proteins, resulting in cell death later in the process. In the main cultivation areas of chickpea like arid and semi-arid regions of the world, Soil salinity is known as a major inevitable constraint [4]. Plants reduce tissue potential by accumulating inorganic and organic solutes for adapting in high salt concentrated soil. Cations Na^+ and K^+ are known to be the major inorganic components of the osmotic potential. Cation Na^+ may cause metabolic disturbances in processes where low Na^+ and high K^+ or Ca_2^+ are required for optimum function [7]. The effect of soil salinity on chickpea is wide ranging and mainly chickpea is affected at germination and early stage [26]. Plant growth regulators are now used on over million hectares worldwide on a diversity of crops each year [22]. Application

of growth promoting hormones is a recent technique in this direction. Plant hormones in a broad sense are organic compounds which play an important role in plant growth development and yield of crops to prevent the fruit and flower drop for a longer period [6]. Plant Growth Promoting Rhizobacteria are groups of bacteria that can actively colonize plant roots and increase plant growth. PGPR can stimulate plant growth, increase yield, reduce pathogen infection and reduce biotic or abiotic plant stress [13]. PGPR either directly helps to provide nutrient to the host plant, indirectly influence root growth and morphology or aide other beneficial symbiotic relationships [14]. Biological approaches such as inoculation of seeds/plants with plant growth promoting bacteria (PGPR) and application of growth regulators to induce resistance against stress have already been attempt. In last few decades, a large array of bacteria including the species of *Pseudomonas, Burkholderia, Agrobacterium, Erwinia, Xanthomonas, Azospirillum, Bacillus, Enterobacter, Rhizobium, Alcaligenes, Arthrobacter, Acetobacter, Acinetobacter, Achromobacter, Aerobacter, Artrobacter, Azotobacter, Clostridium, Klebsiellla, Micrococcus, Rhodobacter, Rhodospirrilum, Flavobacterium* and *Serratia* have been reported to enhance plant growth [18]. In this investigation, the effect of salt stress along with hormones and *acinetobacter* on different varieties of Chickpea (*Cicer arietinum* L.) are studied. The experiment is carried out in pots containing soil which is treated with NaCl at different concentrations. After salt treatment germination percentage is observed and providing treatment of *acinetobacter* and growth hormones for nullifying the salt stress. Seedlings are observed before and after treatment of *acinetbacter* and growth hormones. Morphological and biochemical parameters are observed.

2. Materials and Methods

2.1 Collection of samples

Phule-G-9425-9 and Phule-G-9425-5 varieties of Chickpea were collected from Mahatma Phule Krishi Vidyapeeth, Rahuri, Ahmednagar-413722, and *Acinetobacter tandoii spp.* was collected from the Department of Botany, Savitribai Phule Pune University, Pune.

2.2 Treatment of NaCl to chickpea seedlings

Chickpea seedlings were treated with different salt concentrations (4dS/m, 8dS/m, and 14 ds/m) [25].

2.3 Treatment of *Acinetobacter spp.* and hormones to salt stressed chickpea

Chickpea seedlings were treated by a spread with different NAA and IBA at 5mg/L [21] and the bacterial culture suspension was incubated for 3 days and 5ml of bacterial suspension are spread after incubation [20].

2.4 Morphological parameters

After seven days seed germination percentage was calculated by using the following formula [9]:

$$\text{Germination percentage (\%)} = \frac{\text{No. of germinated seeds}}{\text{Total no. of seeds}} \times 100$$

Root and shoot length of chickpea seedlings were measured under various treatment of salt concentration. After 10, 20 and 30 days interval root and shoot length measured by using the scale. Seed vigor index was calculated by using the following formula: [3]

$$\text{SVI} = \frac{\text{Germination precentage x Total seedling length}}{100}$$

2.5 Biochemical parameters

Amount of total chlorophyll content in control and stress treated seedling was estimated by arnon method and calculated by using following formula: [5]

$$\text{Total chlorophyll (mg/g)} = 20.2(A645) + 8.02\ (A663) \times \frac{V}{1000 \times w}$$

Amount of protein concentration in control and stress treated seedling was estimated by Bradford method: [24]. Amount of proline concentration in control and stress treated seedling was estimated by ninhydrin method and calculated by using following formula: [8]. (Where 115.5 is the molecular weight of proline)

$$\text{Proline } (\mu moles/ml) = \frac{\mu g\ proline/ml}{115.5} \times \text{ml toluene} \times \frac{5}{\text{g sample}}$$

In SDS-APGE analysis, protein was extracted from fresh leaf by using extraction buffer containing Tris HCl- 0.05 M, 0.02% SDS, 30.3% Urea, 1% 2- β mercaptoethanol in a chilled mortar and pestle. The extracted protein was separated out with the help of polyacrylamide gel electrophoresis [16]. In antioxidant Enzyme Assay, 200 mg samples were homogenized in a chilled mortarand pestle with 2 ml of an ice-cold 0.1 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM PMSF and 5% (w/v) PVP. The homogenates was centrifuged at 4°C for 20 min at 15,000rpm. The supernatant fraction was used as crude extract for enzyme activity assays, like superoxide dismutase (SOD) and catalase (CAT) [11]. Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium. One unit of SOD was defined as the amount of enzyme required to cause 50 per cent inhibition of NBT reduction per min at 560 nm [12]. For measurement of catalase enzyme activity, the decline in absorbance was recorded at 240 nm for three min at an interval of 30 sec. The amount of hydrogen peroxide decomposed was determined from molar extinction coefficient (ε36 M^{-1} cm^{-1}). The enzyme activity was expressed as µmoles of H_2O_2 decomposed mg^{-1} protein min^{-1} [2].

Δ O.D. = Initial absorbance – final absorbance

Catalase activity = Δ O.D. x 36 M^{-1}/cm (extinction coefficient)

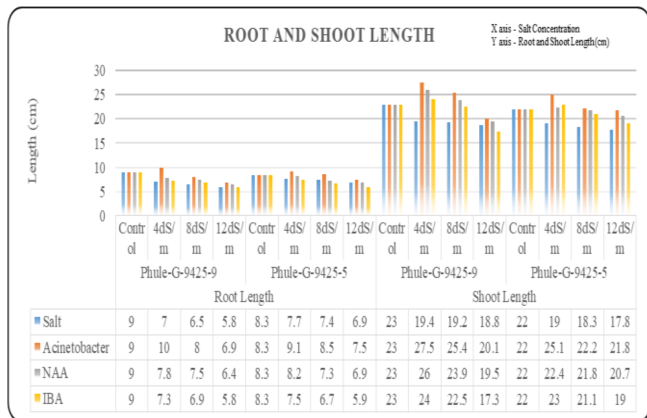
Specific activity (unit/mg) = catalase activity /mg of protein in 0.2 ml of extract.

3. Results and discussion

3.1 Morphological Parameter

The germination percentage was decreased with an increase in NaCl concentrations. Phule-G-9425-9 showed 100% in control, 4dS/m and 8dS/m, in 12ds/m showed 90%. Phule-G-9425-5 showed 100% in control and 4dS/m. where 90% in 8dS/m and 70% in 12dS/m seen. Seed vigor index decreased in responses of increasing salt stress. The root and shoot length of Phule-G-9425-9 and Phule-G-9425-5

decreased with an increase in NaCl concentrations. After 30th days *acinetobacter* and hormones treatments were provided and obtain statistics. In which, *acinetobacter* and hormones treatments were showed positive results on stress induce chickpea. The above-obtained statistics mention in Fig 1.

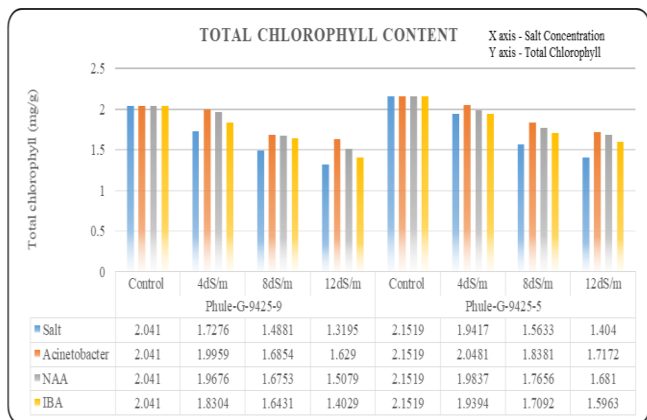


	Root Length								Shoot Length							
	Phule-G-9425-9				Phule-G-9425-5				Phule-G-9425-9				Phule-G-9425-5			
	Control	4dS/m	8dS/m	12dS/m	Control	4dS/m	8dS/m	12dS/m	Control	4dS/m	8dS/m	12dS/m	Control	4dS/m	8dS/m	12dS/m
Salt	9	7	6.5	5.8	8.3	7.7	7.4	6.9	23	19.4	19.2	18.8	22	19	18.3	17.8
Acinetobacter	9	10	8	6.9	8.3	9.1	8.5	7.5	23	27.5	25.4	20.1	22	25.1	22.2	21.8
NAA	9	7.8	7.5	6.4	8.3	8.2	7.3	6.9	23	26	23.9	19.5	22	22.4	21.8	20.7
IBA	9	7.3	6.9	5.8	8.3	7.5	6.7	5.9	23	24	22.5	17.3	22	23	21.1	19

Fig 1: Root and shoot length of Phule-G-9425-9 and Phule-G-9425-5 seedlings.

3.2 Biochemical parameters

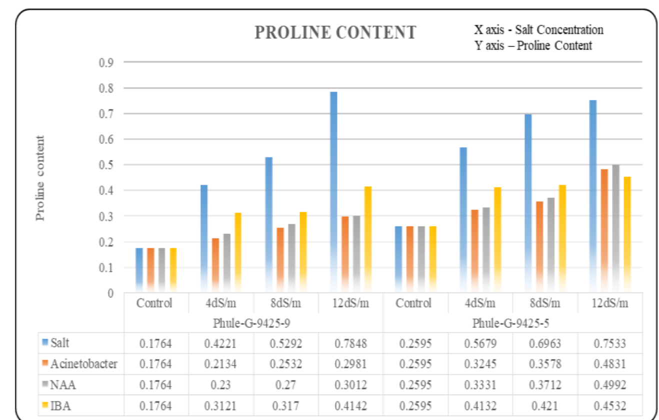
Total chlorophyll content in Chickpea cultivars was decreased with an increase in salt concentrations but after treatment of *acinetobacter* and growth hormones, there was observed increased chlorophyll content. Growth hormones show better results than *acinetobacter*. NAA and IBA showed high total chlorophyll content than *acinetobacter* and salt-stressed plants. It may be the *acinetobacter* and growth hormones, which help to regain the total chlorophyll content in salt-stressed plants. The above-obtained statistics mention in Fig 2.



	Phule-G-9425-9				Phule-G-9425-5			
	Control	4dS/m	8dS/m	12dS/m	Control	4dS/m	8dS/m	12dS/m
Salt	2.041	1.7276	1.4881	1.3195	2.1519	1.9417	1.5633	1.404
Acinetobacter	2.041	1.9959	1.6854	1.629	2.1519	2.0481	1.8381	1.7172
NAA	2.041	1.9676	1.6753	1.5079	2.1519	1.9837	1.7656	1.681
IBA	2.041	1.8304	1.6431	1.4029	2.1519	1.9394	1.7092	1.5963

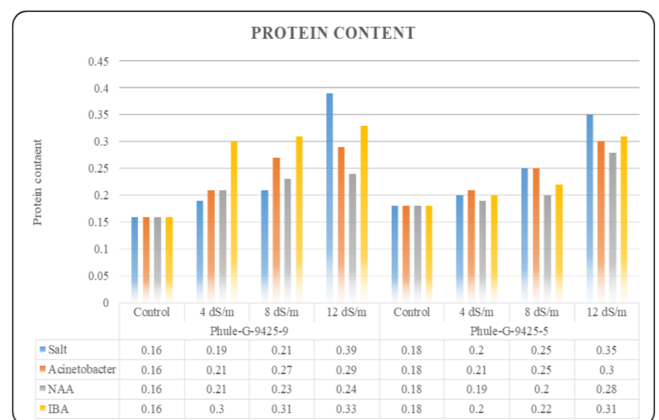
Fig 2: Total chlorophyll content of Phule-G-9425-9 and Phule-G-9425-5 seedlings.

As the NaCl concentrations were increased there was increased in proline content in both cultivars of chickpea. In Phule-G-9425-5 it was found higher than Phule-G-9425-9. Protein content decreased up to 8 dS/m but again little bit increment was seen in 12 dS/m salt concentrations. But after the treatment of *acinetobacter* and growth hormones, there was observed decreased proline content. Protein content become lower was seen. The above-obtained statistics mention in Figures 3 and 4.



	Phule-G-9425-9				Phule-G-9425-5			
	Control	4dS/m	8dS/m	12dS/m	Control	4dS/m	8dS/m	12dS/m
Salt	0.1764	0.4221	0.5292	0.7848	0.2595	0.5679	0.6963	0.7533
Acinetobacter	0.1764	0.2134	0.2532	0.2981	0.2595	0.3245	0.3578	0.4831
NAA	0.1764	0.23	0.27	0.3012	0.2595	0.3331	0.3712	0.4992
IBA	0.1764	0.3121	0.317	0.4142	0.2595	0.4132	0.421	0.4532

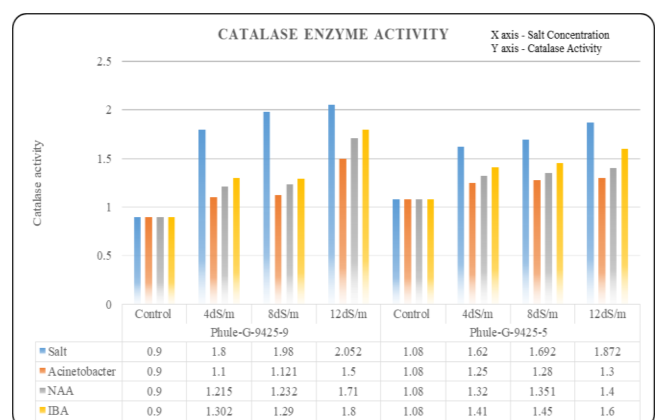
Fig 3: Proline content of chickpea cultivars.



	Phule-G-9425-9				Phule-G-9425-5			
	Control	4 dS/m	8 dS/m	12 dS/m	Control	4 dS/m	8 dS/m	12 dS/m
Salt	0.16	0.19	0.21	0.39	0.18	0.2	0.25	0.35
Acinetobacter	0.16	0.21	0.27	0.29	0.18	0.21	0.25	0.3
NAA	0.16	0.21	0.23	0.24	0.18	0.19	0.2	0.28
IBA	0.16	0.3	0.31	0.33	0.18	0.2	0.22	0.31

Fig 4: Protein content in chickpea cultivars.

In the antioxidant enzyme assay, there were catalase and SOD. Both antioxidant enzymes increased in chickpea when salt stress was increased. In stress-induced Phule-G-9425-9 and Phule-G-9425-5 provided treatments of *acinetobacter* and growth hormones, after that, they showed less catalase and SOD activity. Detail statistics are presented in Figures 5 and 6.



	Phule-G-9425-9				Phule-G-9425-5			
	Control	4dS/m	8dS/m	12dS/m	Control	4dS/m	8dS/m	12dS/m
Salt	0.9	1.8	1.98	2.052	1.08	1.62	1.692	1.872
Acinetobacter	0.9	1.1	1.121	1.5	1.08	1.25	1.28	1.3
NAA	0.9	1.215	1.232	1.71	1.08	1.32	1.351	1.4
IBA	0.9	1.302	1.29	1.8	1.08	1.41	1.45	1.6

Fig 5: Catalase content in chickpea cultivars.

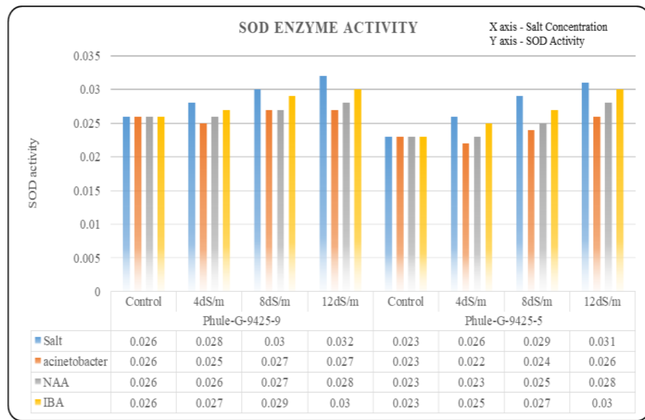
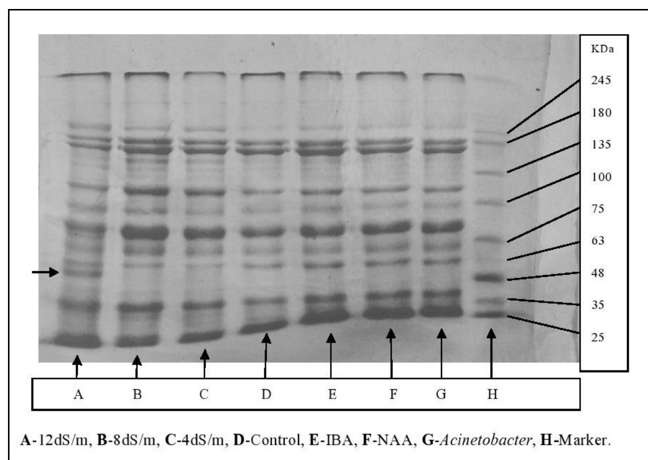


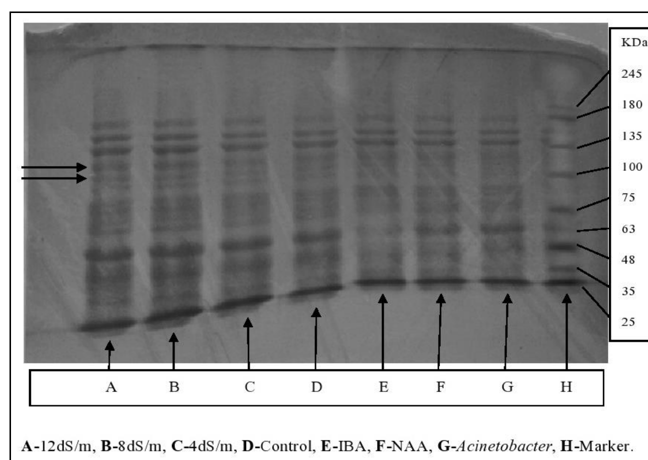
Fig 6: SOD content in chickpea cultivars.

The protein was extracted from Phule-G-9425-9 and Phule-G-9425-5 and seen in SDS-PAGE. In both salt-stressed induced chickpea cultivars, extra protein bands were seen. Extra protein bands were saw in Phule-G-9425-9, which was in the range of 48 and 63 KDa. In Phule-G-9425-5 saw in the range of 135 and 100 KDa. But after the treatment of *acinetobacter* and growth hormone, those bands were disappeared. Stress-induced chickpea treated with *acinetobacter* and growth hormones does not show this kind of protein banding. Protein profiling is presented in Figure 7 and 8.



A-12dS/m, **B**-8dS/m, **C**-4dS/m, **D**-Control, **E**-IBA, **F**-NAA, **G**-*Acinetobacter*, **H**-Marker.

Fig 7: Protein profiling of Phule-G-9425-9



A-12dS/m, **B**-8dS/m, **C**-4dS/m, **D**-Control, **E**-IBA, **F**-NAA, **G**-*Acinetobacter*, **H**-Marker.

Fig 8: Protein profiling of Phule-G-9425-5

4. Conclusion

The above results finally concluded that chickpea cultivars are majorly affected by salt stress on the morphological and biochemical levels. Therefor *Acinetobacter* and growth hormones are play important role in chickpea cultivars. *Acinetobacter* and growth hormones are responsible for nullifying the effect of salt stress. Those are able to maintain catalase, SOD, Chlorophyll content, proline, and protein content, which was unstable by salt stress. Majorly NAA has nullified the stress effect more prominently.

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6. Conflict of interest

All authors declare that they have no conflict of interest.

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