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The Combined Effects of Gibberellic Acid and *Rhizobium* on Growth, Yield and Nutritional Status in Chickpea (*Cicer arietinum* L.)

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Abstract: Plant growth regulators and *Rhizobium* are actively involved in the regulation of flowering, pod formation, nodulation, and ultimately the growth and yield of legumes. However, very limited information is available on the combined effect of gibberellic acid (GA_3) and *Rhizobium* on growth attributes and yield of legume crops. This experiment was designed to fill this gap by studying the performance of chickpea under exogenous application of GA_3 (10^{-4} and 10^{-5} M) alone and in combination with *Rhizobium*. Exogenous application of GA_3 (10^{-5} M) combined with rhizobium inoculation gave the highest values for number of nodules per plant (16) and their dry biomass (0.22 g). Moreover, GA_3 application and seed inoculation with *Rhizobium*, when applied singly, significantly enhanced chickpea growth. However, the most promising results were obtained by the inoculation of *Rhizobium* accompanied with GA_3 (10^{-5} M). Plant height, grain and stover yield, and chlorophyll contents were enhanced up to 35%, 39%, 21%, and 51%, respectively. Likewise, the bioaccumulation of macronutrients (N, P and K) was maximum in plants receiving both *Rhizobium* inoculation and GA_3 application. It is concluded that the combined application of *Rhizobium* and GA_3 has synergistic effects on the growth, yield, and nutrient contents of chickpea.

Keywords: nutritional quality; nodulation; PGRs; *Rhizobium*; crop production; chickpea

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1. Introduction

Agricultural revolution and crop intensification are among the recent challenges to supply food and fiber for ever increasing human population [1,2]. However, poor soil fertility and low fertilizer use efficiency hinders sustainable crop production [3,4]. It has now become a great dilemma for the scientific community to increase per hectare yield and utilization of existing arable lands to sustain food production. For this, the use of chemical fertilizers has gained popularity among farmers to improve soil fertility and increase crop yields [5]. At present, about 40–60% crop (cereal) production relies on synthetic fertilizers and it is estimated that by 2050 the crop production will have to rely on fertilizers up to 110% [6]. The use of inappropriate agro-technology by the farmers on the other hand has further excavated this problem in the form of environmental pollution, deterioration of soil quality and loss of biodiversity [7,8]. This is especially true for Pakistan, where the unwise use of agrochemicals,

imbalanced fertilization, utilization of untreated wastewater and farm wastes are in common practice for agricultural production [9]. There is a significant difference between the quantity of applied fertilizers and that utilized by the plants. Only a small fraction of the applied fertilizer is utilized by the plants and the rest is lost in the environment and hence causes negative effects on the environment. Therefore, the application of alternative approaches that are environmentally sound, cost effective, and farmer friendly are required to overcome these constraints both agronomically and economically [10].

Chickpea is the most vital grain legume of Leguminosae family cultivated around the world and an important source of fiber, oil, fat, ash and protein [11]. Biological nitrogen fixation (BNF) is a physiological process that facilitates agricultural crop production by adequate N supply [12]. Being a leguminous plant, chickpea establishes a symbiotic relationship with rhizobia present in its root nodules and thus support BNF [2]. These plants are associated with an array of bacteria having the ability to reduce atmospheric (inert N_2) into a form available for plants through BNF [13]. A lack of suitable rhizobial strains causing poor nodulation, flower drop and pod shedding [14] is among the main causes of lower production of chickpea [15,16]. In soils, deprived of suitable rhizobial strains, the artificial inoculation of chickpea seed is an effective approach for enhancing root nodulation and ultimately yield [2,17]. It is well established that rhizobia associate with or adhere nearby the roots of legumes and promote growth through phosphate solubilization, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, siderophore production, indole-3-acetic acid (IAA) production, catalase, oxidase, etc. [18–21].

Root nodulation is a complex and sensitive phenomenon requiring synchronized bacterial infection, plant organogenesis and root growth [22–24]. Signaling pathways of nodulation factors are stimulated through Ca^{2+} nuclear oscillations and spiking to initiate root nodulation in cortical cells [25,26]. Almost all signals and organogenesis measures are extensively affected by the hormones balance in plants [27]. These hormones affect root nodulation and nitrogen fixation in legumes both positively and negatively. The stimulating effects of phytohormones, i.e., auxins and cytokinins in root nodulation are well documented. Moreover, auxins, cytokinins, and gibberellins (gibberellic acids, GAs), through crosstalk with cytokinins signaling paths, regulate the root nodulation in legumes [28].

Legume–*Rhizobium* symbiosis is mostly reliant on environmental contexts, soil fertility, and plant growth stages. Owing to these factors, plants may be incapable to biosynthesize adequate amount of these phytohormones endogenously. Exogenous application of these phytohormones results in enhanced crop yield by regulating pod formation and flower drop [16,29]. The plants respond to exogenous application of phytohormones and among plant hormones, GA_3 is an important growth hormone and exogenous application of GA_3 in legumes regulates nodule formation [28].

The nodulation process is sensitive to environmental fluctuations and further subject to soil conditions that might affect nodule formation. In this regard, the exogenous application of gibberellic acid (GA_3) could be an important strategy to support nodulation process and sustain crop production. Moreover, the combined use of gibberellic acid (GA_3) and *Rhizobium* sp. has already been shown to improve growth, physiology and yield of crops [30]. Various reports are available where alone application of *Rhizobium* or plant growth regulators (PGRs) have been investigated to enhance growth and yield of various leguminous and non-leguminous crops [2,31–34]. However, their combined effects on crops have rarely been studied. Taking this in view, we hypothesized that combined application of gibberellic acid (GA_3) and rhizobia may enhance growth, nodulation and yield of chickpea, while their effects may vary based on the concentration levels. Therefore, the aim of the present study was to evaluate the combined effect of *Rhizobium* sp. and exogenously applied GA_3 on growth, root nodulation, and ultimately the yield of chickpea and to evaluate the possible correlations among applied treatments and studied parameters.

2. Materials and Methods

2.1. Collection of Nodulated Chickpea Roots and Isolation of Rhizobium Species

Several healthy nodulated plants of chickpea were collected from diverse fields of Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. These plants were uprooted carefully and selected, based on pink colored healthy nodules, transported to the Soil Bacteriology Section, Agri. Biotechnology Research Institute, Faisalabad, Pakistan in polythene bags.

Rhizobium sp. was isolated in yeast extract mannitol agar (YEMA) medium [35]. Pink, firm, healthy, and unbroken nodules were rinsed under sterilized water to eliminate adhering soil particles. Nodules surface sterilization process was carried out by dipping in 70% ethyl alcohol, followed by repeated washing in sterilized water, and then a 3% sodium hypochlorite solution was used for further surface sterilization [36]. The nodules were surface washed three times with sterile water and crushed in a crucible. Suspension of crushed nodules was plated on the YEMA medium having 1% Congo-red dye followed by 24 h incubation at 28 ± 2 °C. Purified growth was shifted to the broth medium of yeast mannitol (YM) and kept in a shaker (revolving at 75 rpm) at 28 ± 2 °C and preserved in the refrigerator at 4 °C.

2.2. Identification and Phenotypic Characteristics of Isolates

Ten pure isolated cultures were made to perform Gram reaction and then subjected to biochemical tests and morphological traits. The potential of rhizobial cultures to grow on various salt concentrations was examined by streaking on YEM broth with 0.5, 1, 1.5, 2, and 2.5% (*w*/*v*) NaCl [37].

The ability to grow on different pH levels of these bacterial isolates was determined by adjusting pH values between 4 and 9 in triplicates [38]. Glucose peptone agar and lactose assays were used to test the ability of the microbes for glucose and lactose utilization as a sole carbon source. Furthermore, biochemical tests such as gelatin hydrolysis, fluorescence assay, triple sugar ion agar, lipase [39], lysine decarboxylase, citrate, urease, starch hydrolysis, and bromothymol blue tests [40] were also performed.

The selected isolates of *Rhizobium* sp. were identified and confirmed through Biolog® identification system (Microlog System Release 4.2; Biolog Inc., Hayward, CA, USA) (Table 1). The Biolog® identification system is equally effective as 16S rRNA gene sequencing for identification of *Rhizobium* sp. [41].

2.3. Detection of IAA and GA in Broth Culture

Auxin production of *Rhizobium* was determined calorimetrically in terms of IAA equivalents. Five days old starter cultures of *Rhizobium* were used in Yeast Mannitol broth (150 mL) and placed on a shaker revolving at speed of 75 rpm at 28 ± 2 °C for 72 h followed by centrifugation (10,000 rpm for 20 min). After centrifugation, 3 mL of supernatants were used with 2 mL of Salkowski's reagent, i.e., 2 mL 0.5 M $FeCl_3$ and 98 mL 35% $HClO_4$. This mixture was incubated at room temperature (30 min). Spectrophotometric absorbance was recorded at 535 nm. The standardized curve [42] was used to determine auxin concentration. The isolates giving the highest values of auxin were selected for further study.

The gibberellin contents were measured calorimetrically by using a standard procedure [43] with slight modifications. In this method, 2 mL zinc acetate reagent were added in 15 mL of supernatant. After 2 min, 2 mL potassium ferrocyanide was added in the mixture followed by centrifugation for 15 min (2000 rpm). After centrifugation, 5 mL HCl (30%) was added in the supernatant (5 mL) and incubated for 75 min at 20 °C. A standard curve was prepared using GA_3 (Hi-media) in concentrations ranging from 100 to 1000 μg m^{-1}. Spectrophotometric absorbance was recorded at 254 nm. In the case of blank, 5% HCl was used.

Table 1. Physiochemical and growth promoting parameters of *Rhizobium.*

Characteristics	Rhizobial Isolates									
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**
Phenotypic and physiological characterization										
Colony color	White	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Size (mm)	2.5	3.1	2.1	1.8	3.0	2–3	2.6	2–4	2–4	2.5
Opacity	Transparent	Transparent	Transparent	Translucent	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent
Gram reaction	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Bacterium shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility test	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Bacterial growth conditions [a,b]										
NaCl										
0.5%	++	+	+	++	++	++	++	++	+	+
1.0%	++	+	+	+	++	++	++	++	++	++
1.5%	+	+	+	+	+	++	++	++	++	+
2.0%	+	++	+	+	++	+	++	++	++	+
2.5%	++	++	++	++	+	+	++	+	+	++
pH										
4	−	+	+	−	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	−	+
6	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	−	+	−
9	−	−	+	+	+	−	+	+	+	+
Carbon utilization potential										
GPA assay	+	−	+	+	+	+	+	+	+	+
Lactose Assay	−	+	−	−	−	−	−	−	−	−
Biochemical Characterization										
Gelatin Hydrolysis	−	−	−	−	−	−	−	−	−	−
Fluorescence assay	−	−	−	−	−	−	−	−	−	−
Triple sugar Ion	+	+	+	+	+	+	+	+	+	+
Lipase	−	+	+	+	+	+	+	−	−	+
Lysine	+	+	+	+	+	+	−	+	−	+
Citrate	+	+	+	+	+	+	+	−	+	+
Urease	+	+	+	+	+	+	+	+	+	−
Starch Hydrolysis	−	−	−	+	+	+	−	−	+	+
Bromothymol blue	+	+	+	+	+	+	+	+	+	+
Auxin (IAA equivalents µg mL^{-1})	4.34	5.67	6.86	4.23	3.01	2.34	4.45	5.68	4.21	2.47
Gibbrellic acid (mg L^{-1})	5.37	8.34	4.87	11.36	7.42	7.87	6.11	8.75	8.99	7.01
	Biolog identification and similarity index %									
Mesorhizobium ciceri	76	80	74	76	74	73	72	78	76	74

[a] −, absent; +, present [b] +, low efficiency; ++, high efficiency.

2.4. Seed Bacterization of Cicer Arietinum L.

The inoculum of selected Rhizobial isolate R4 (based on high GA production and other growth promoting traits) was prepared in 200 mL Erlenmeyer flask containing yeast extract mannitol (YEM) medium (Yeast, 0.5 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; distilled water, 1000 mL; pH, 6.8), incubated at 28 °C in a shaking incubator at 160 rpm for 2 days. An optical density of 0.5 ($OD_{0.5}$), measured with a spectrophotometer at λ 600 nm, was achieved by dilution to maintain a uniform cell density (10^8 CFU mL^{-1}) prior to seed inoculation. Surface sterilized healthy seeds were inoculated with *Rhizobium* broth (10 mixed with 10% jaggery solution and sterilized peat. Seeds were mixed thoroughly until a fine layer of inoculum appeared on them and spread on the polythene sheet to dry in the laboratory overnight.

2.5. Pot Experiment

Effect of *Rhizobium* inoculant alone or combined with two different concentrations of GA_3 i.e., 10^{-4} M and 10^{-5} M on growth and yield parameters of chickpea was studied in a wire house pot experiment at the research area of the Soil Bacteriology Section, AARI, Faisalabad, Pakistan. The soil used for the experiment was air-dried, thoroughly mixed and sieved (2 mm). The soil was sandy clay loam having 7.89 pH, 1.36 dS m^{-1} EC, 0.77% organic matter, 0.034% total nitrogen, 7.66 mg kg^{-1} available phosphorus 112 mg kg^{-1} extractable potassium. Each pot contained 16 kg soil.

The research experiment contained the following six treatments: (1) Control, (2) *Rhizobium* inoculation, (3) GA_3 (10^{-4} M), (4) GA_3 (10^{-5} M), (5), *Rhizobium* + GA_3 (10^{-4} M), (6), and *Rhizobium* + GA_3 (10^{-5} M). Surface sterilized chickpea seeds were inoculated with *Rhizobium* sp. R4 using slurry as described previously. In control, seed was coated with sterilized LB broth slurry. Four chickpea seeds were sown in each pot and later thinned to two plants after 7 days of sowing (DAS). The trial was conducted in CRD design with four replications. Recommended dosages of chemical fertilizers (N, P & K) were applied.

2.6. Agronomic Traits

Agronomic parameters like plant height, pods $plant^{-1}$, nodules $plant^{-1}$, nodule's weight, and yield (grain and stover) were observed before and after harvesting chickpea plants. For nodulation data, one plant from each replicate was pulled out, with roots, washed in distilled water and mature nodules per plant were weighed and the average was computed. At harvesting (100 DAS after seedling emergence) pods from each plant were taken, counted, and threshed to get grain yield, and the average was worked out. Fifty seeds weight was calculated from the seed lot of each treatment and then mean value was computed.

2.7. Physiological Traits

Physiological attributes were recorded at mid-day (between 10:00 and 14:00) after 60 DAS. For the determination of chlorophyll contents, fully green leaves were selected for the measurement of photosynthetic pigments. To determine chlorophyll contents (a and b), plant leaves were crushed in acetone. The mixture was centrifuged for 10 min at 1000 rpm and absorbance at 645 nm for chlorophyll "a" and 663 nm for chlorophyll "b" was recorded on spectrophotometer [44].

To determine the volume of pink bacteroid tissue of nodules, root nodules were taken at the flowering stage and a minor section of nodules (5 µL) made by using sharp. The amount of pink bacteroid tissue comprising of the leghaemoglobin present in the nodule cortex was noted following the method described by Sadasivan and Manickam [45].

2.8. Physicochemical Analysis of Plant and Soil

Pre and post sowing analysis of soil regarding N, P, and K was performed following the procedure of Mehlich and Mehlich [46]. After harvesting, the representative samples of seeds and stovers were oven dried for 24 h at 65 °C and ground. Nitrogen contents were

determined by micro Kjeldahl's method, phosphorus contents were measured by yellow method and K content by Flame photometric method described by Jackson [47].

Protein and carbohydrate contents in the dry seeds were estimated as described by Lowry et al. [48].

2.9. Statistical Analysis

The collected data regarding growth, yield, and biochemical parameters were subjected to analysis of variance [49]. The significance of differences among treatment means was tested through LSD test (IBM SPSS Statistics 19, USA). Directional relationship among observed parameters and treatments were estimated through Pearson correlation and principal component analysis (R Software® 4.0.2).

3. Results

3.1. Biochemical Characterization

The microscopic studies showed that colonies were rod-shaped gram-negative. All isolates were non-spore forming and motile rods. The colonies of all isolates were circular, 2–4 mm, milky white, transparent (Table 1). Some *Rhizobium* strains can grow under high salt concentration. In the present investigation, it was found that all the strains showed growth at 0.5% (*w*/*v*) NaCl and continued grown up to 2.5% (*w*/*v*) NaCl concentrations. All isolates could grow in the yeast extract mannitol agar (YEMA) medium with pH range 4–9. In the present study, optimum pH for *Rhizobium* isolates growth was found to be 6–7. The minimal growth of isolates was exhibited at pH 4 and pH 9. All isolates were streaked on Bromothymol blue added YEMA selective media for further confirmation. All four strains showed growth in two days and turned YEMA media from blue to yellow, confirming their nature of being fast growers and acid producers. A starch hydrolysis assay was examined to determine the production of reducing sugar from starch in bacteria. The clear zone around colonies was observed after the addition of iodine. Lysine decarboxylase test was performed using Bromocresol purple Falkow media. Results showed a change in the color of medium inoculated with *Rhizobium* sp. Lipase Test was found to be positive for *Rhizobial* isolates R2-R7 and R10. Glucose, sucrose, and starch proved equally good for growth of strains under study (Table 1). *Rhizobial* cells growth on GPA medium depicted the capability of *Rhizobium* to use Glucose as Carbon source (Table 1). However, no growth was observed in case of lactose. Moreover, the solidification of gelatin medium at 4 °C for 30 as well as 60 min depicted that *Rhizobial* cells didn't produce gelatinase. Furthermore, *Rhizobium* cells failed to fluoresce on King's medium under the UV (Table 1). Production of indole acetic acid (IAA) was common to all *Rhizobium* isolates, but the highest value was recorded in R_3 (6.86 µg mL^{-1}). Moreover, in the case of gibberellic acid, maximum concentration was produced by R_4 (11.36 mg L^{-1}). Biolog identification showed that all isolates exhibited similarity to *Mesorhizobium ciceri.* However, the maximum similarity index (80%) with *Mesorhizobium ciceri* was recorded in the case of R_2 isolate compared to the others.

3.2. Growth and Yield Parameters

Growth and yield parameters of chickpea were increased significantly with foliar GA_3 application and *Rhizobium* inoculation either alone or combined (Figures 1 and 2).

Rhizobium inoculation significantly enhanced the plant height, number of nodules $plant^{-1}$, number of pods $plant^{-1}$ grain yield, and fresh and dry weight of nodules over the control (Figures 1 and 2). Foliar spray of GA_3 on *Rhizobium* inoculated plants had an additive effect on plant height, nodules $plant^{-1}$, and fresh and dry weight of nodules by 35, 55%, 16%, and 12%, respectively (Figure 1). *Rhizobium* supplemented with GA_3 (10^{-5} M) gave the maximum increase in stover biomass, i.e., 21%, compared to control. Statistically significant increase in grain yield was recorded with the alone applications of *Rhizobium* and GA_3 (10^{-5}, 10^{-4} M). *Rhizobium* seed treatment enhanced grain yield up to 10% over control (Figure 1). The combined application of *Rhizobium* and GA_3 (10^{-4} M) increased

the yield by 39% than control. The least increase in grain yield i.e., 9%, was recorded with the alone application of GA_3 (10^{-4} M). *Rhizobium* inoculation supplemented with foliar application of GA_3 increased the pods plant^{-1} and dry pod weight. Maximum increase (41% and 57%, respectively) was recorded for GA_3 at 10^{-4} M on *Rhizobium* inoculated treatments (Figure 2).

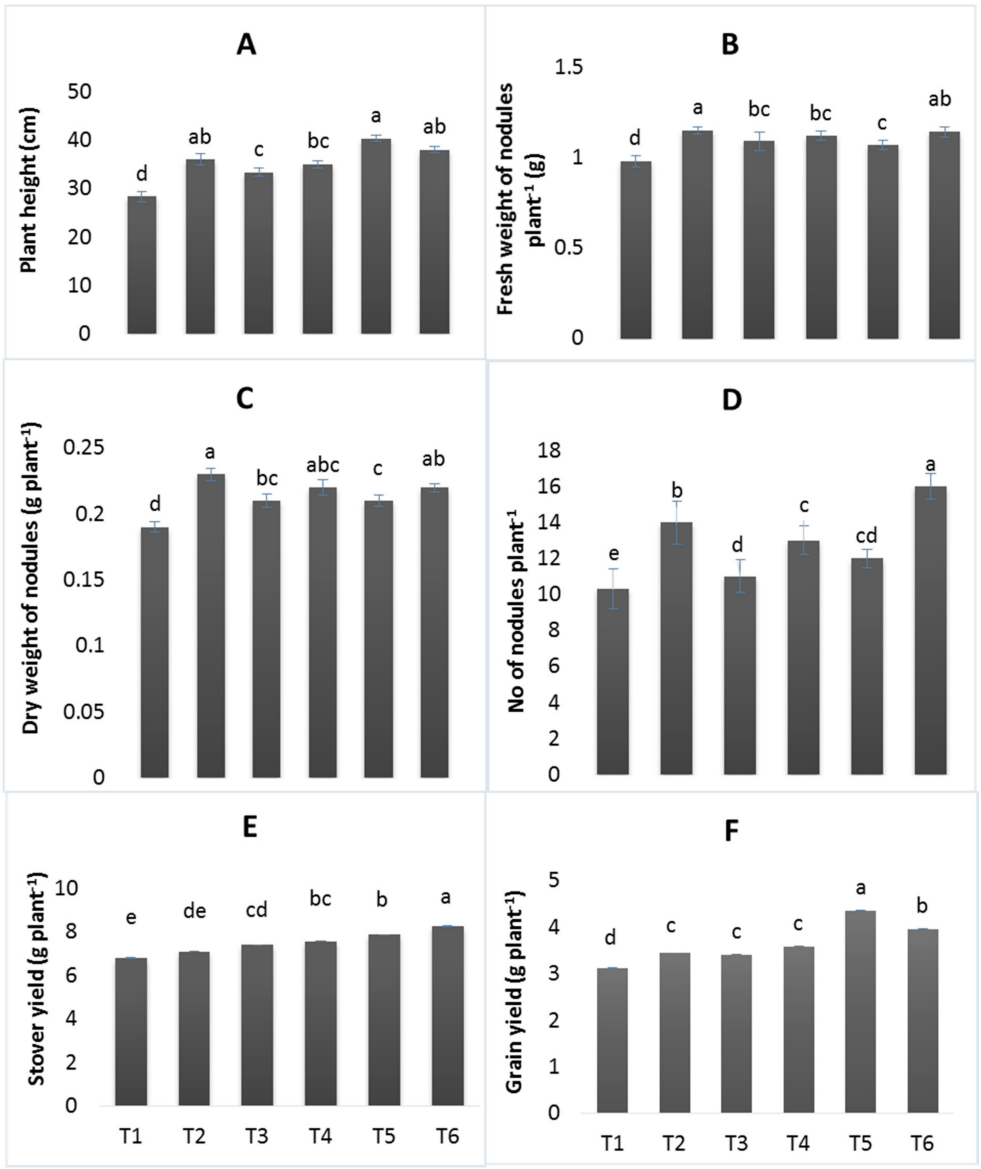


Figure 1. Effect of plant growth hormone and *Rhizobium* sp. On growth (**A**,**B**), nodulation (**C**,**D**) and yield (**E**,**F**) attributes of chickpea. T1: untreated control, T2: *Rhizobium* sp. Inoculation, T3: GA3 (10^{-4} M), T4: GA3 (10^{-5} M), T5: *Rhizobium* + GA3 (10^{-4} M), T6: *Rhizobium* +GA3 (10^{-5} M). Quantities sharing similar letters are not statistically different from each other at $p > 0.05$.

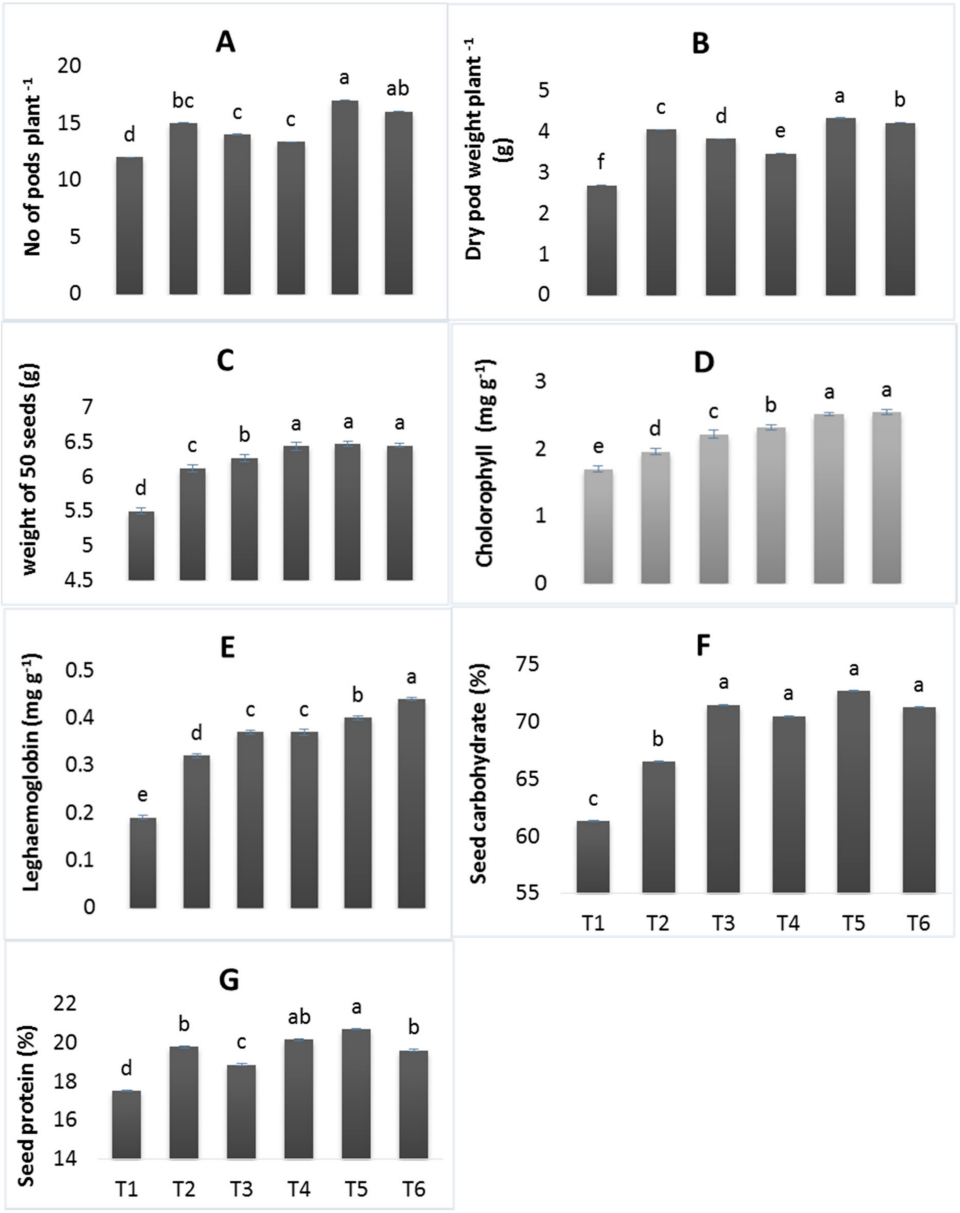


Figure 2. Effect of plant growth hormone and *Rhizobium* sp. On some yield (**A**–**D**) and quality (**E**–**G**) parameters of chickpea. T1: untreated control, T2: *Rhizobium* sp. Inoculation, T3: GA3 (10^{-4} M), T4: GA3 (10^{-5} M), T5: *Rhizobium* + GA3 (10^{-4} M), T6: *Rhizobium* +GA3 (10^{-5} M). Quantities sharing similar letters are not statistically different from each other at $p > 0.05$.

3.3. Physiological and Quality Parameters

Rhizobium inoculation along with foliar GA_3 application positively increased the chlorophyll a and b contents (Figure 2). *Rhizobium* and GA_3 (10^{-5} M) combined gave the maximum increase of 51%. Alone application of *Rhizobium* resulted in an increase of 15% in chlorophyll contents over control.

It was observed that the highest leghaemoglobin contents (0.40 mg/g) were recorded with the combined application of GA_3 (10^{-5} M) with *Rhizobium* inoculation in comparison to the control treatment (Figure 2).

Data regarding seed carbohydrate and protein contents were positively increased by the inoculation of *Rhizobium* but this response was further enhanced with GA_3 application. The highest increase of seed carbohydrate (16%) and protein contents (12%) were recorded for GA_3 (10^{-4} M) with *Rhizobium* inoculation (Figure 2).

3.4. Chemical Analysis of Soil and Plant

Application of *Rhizobium* or GA_3 improved nitrogen (N), phosphorous (P), and potassium (K) contents individually (Figure 3). A higher increase in stover N, P, and K contents by 18%, 47%, and 15% was attained with the mutual application of *Rhizobium* and GA_3 (10^{-5} M). Similarly, *Rhizobium* along with GA_3 (10^{-5} M) enhanced seed N, P, and K contents by 4%, 21%, and 34%, respectively (Figure 3). Foliar GA_3 application and *Rhizobium* both increase nutrient contents when applied individually. Maximum gain in N, P, and K contents (0.037%, 8.03 mg kg^{-1}, 119 mg kg^{-1}) was recorded application of GA_3 (10^{-5} M) and *Rhizobium* (Figure 3).

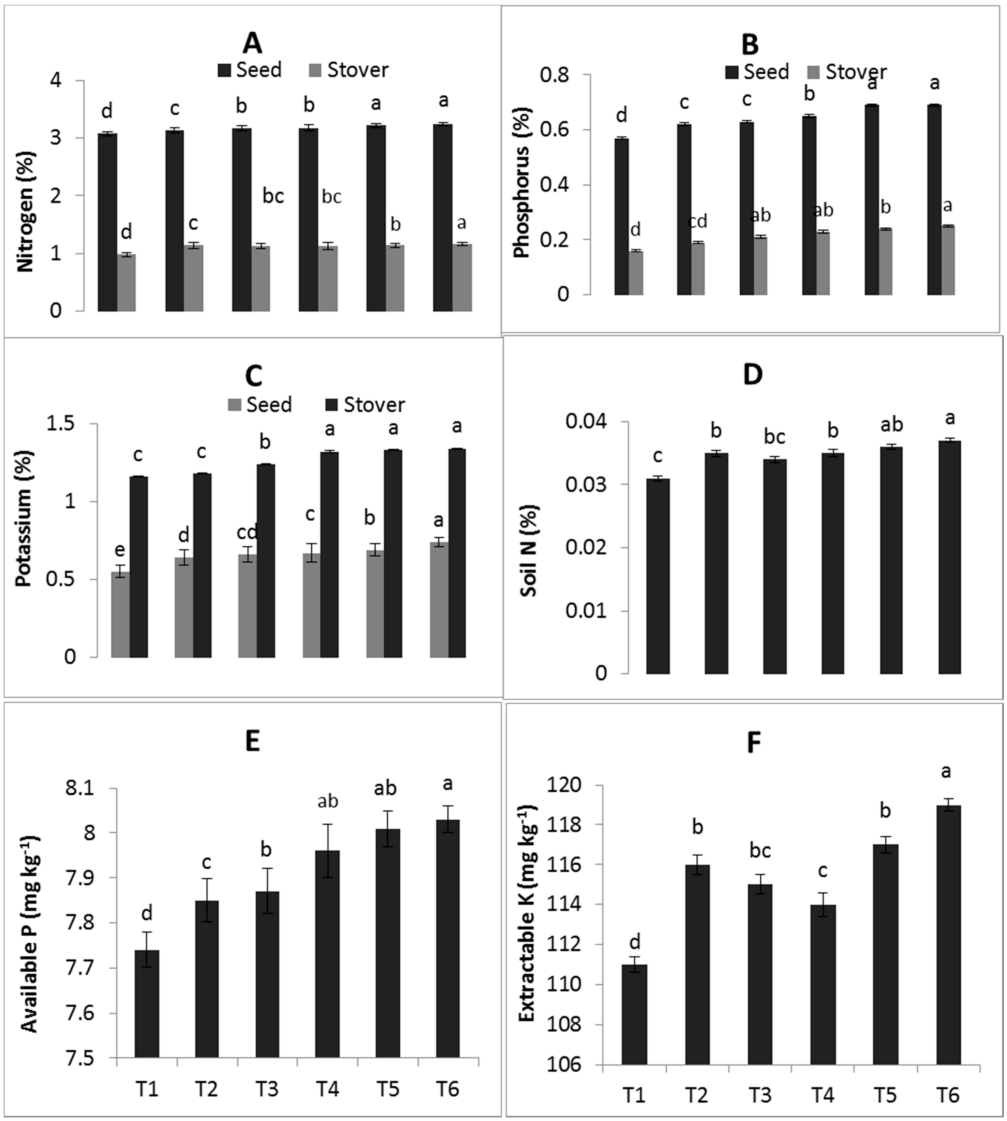


Figure 3. Effect of plant growth hormone and *Rhizobium* sp. On plant (**A**–**C**) and soil (**D**–**F**) nutrient contents of chickpea. T1: untreated control, T2: *Rhizobium* sp. Inoculation, T3: GA3 (10^{-4} M), T4: GA3 (10^{-5} M), T5: *Rhizobium* + GA3 (10^{-4} M), T6: *Rhizobium* + GA3 (10^{-5} M). Quantities sharing similar letters are not statistically different from each other at $p > 0.05$.

3.5. Pearson Correlation and Principal Component Analysis

All agronomic traits, i.e., plant height, nodules weight (fresh and dry), pod dry weight, pods number, and yield (grain and stover) exhibited strong positive correlation with physiological parameters (chlorophyll contents), biochemical attributes (leghemoglobin contents, seed carbohydrate, protein contents), and mineral contents of the plant (stover K, seed N, stover P, seed P, stover N, and seed K) (Figure 4).

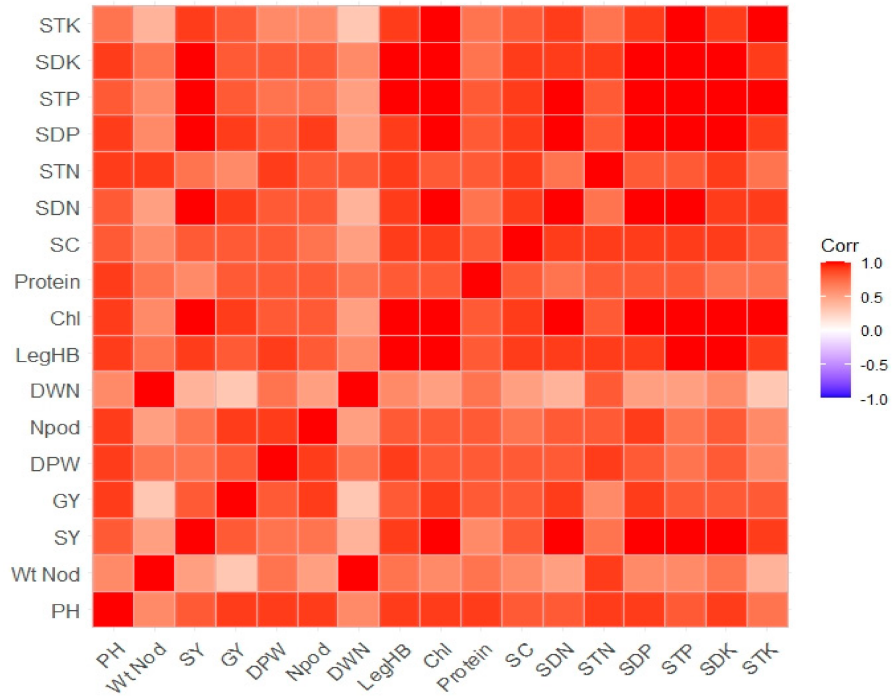
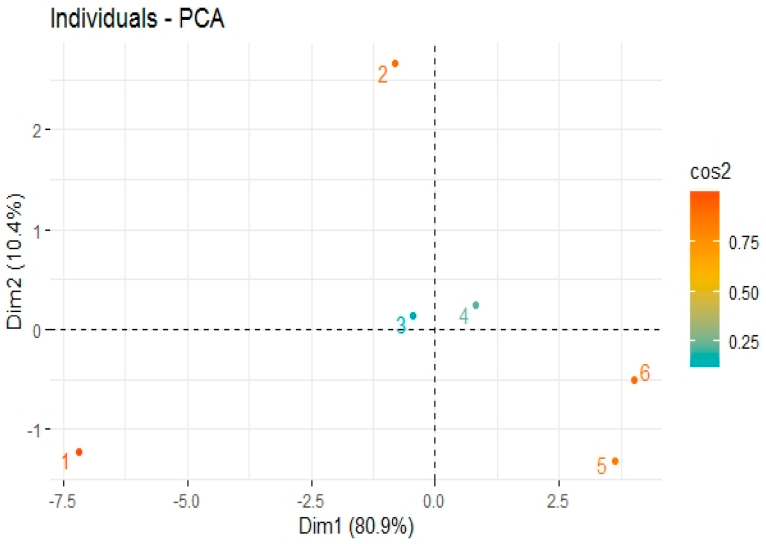
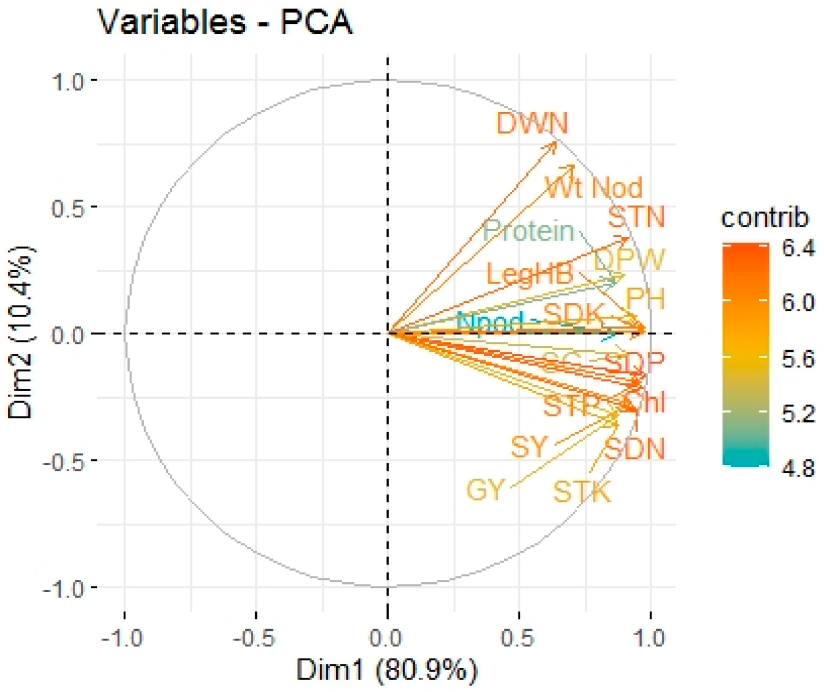


Figure 4. Represents correlation among measured parameters, where abbreviations of correlation matrix are as Grain Yield (GY), Stover Yield (SY), Chlorophyll contents (Chl), Number of pods (N pod), Seed carbohydrate contents (SC), Plant height (PH), Leghaemoglobin contents (Leg HB), Dry pod Weight (DPW), Protein contents (Protein), Weight of nodules (Wt Nod), dry weight of nodules (DWN), Stover K (STK), Seed N (SDN), Stover P (STP), Seed P (SDP), Stover N (STN), and Seed K (SDK). Dark red = highly positive, Light red = less positive.

The score and biplots for some important traits of chickpea are shown in Figure 5. Of the extracted components, PC_1 and PC_2 explained maximally and accounted for 91.3% of total observed variation in the dataset. The first component PC_1 accounted for (80.9%) while the second component accounted for (10.4%) of the total variance (Figure 5A,B). Thus, both of these components accounted for most of the positive effects of combined application of GA_3 and *Rhizobium* on growth and yield of chickpea plants. Principle component analysis further confirmed that growth hormone alone or in combination with *Rhizobium* significantly improve studied attributes of chickpea plants. All the six applied treatments were successfully separated by the principal components (Figure 5A). The control, *Rhizobium* inoculation and GA_3 (10^{-4} M) were clustered in PC_1 whereas, GA_3 (10^{-5} M), *Rhizobium* + GA_3 (10^{-4} M) and *Rhizobium* + GA_3 (10^{-5} M) were clustered in PC_2. This distribution of treatments showed combined application of GA_3 and *Rhizobium* had positive effects on growth and yield determining attributes of chickpea (Figure 5A). Moreover, the vectors representing the tested attributes of chickpea plants were displaced away from the point of origin and reached to the edges, further indicating that there exists a strongly positive correlation among these variables as indicated by the sharp angles of vectors (located close to each other) (Figure 5B). PC_1 was defined as a collective function of grain yield (GY), stover yield (SY), chlorophyll contents (Chl), number of pods (N pod), seed carbohydrate contents (SC), plant height (PH), leghaemoglobin contents (leg HB), dry pod Weight (DPW), protein contents (protein), weight of nodules (Wt Nod), dry weight of nodules (DWN), stover K (STK), seed N (SDN), stover P (STP), seed P (SDP), stover N (STN), and seed K (SDK).



(**A**)



(**B**)

Figure 5. Score plots (**A**) and loading plots (**B**) of PCA on different attributes of chickpea by application of plant growth hormone alone and in combination with *Rhizobium sp*. Score plots (**A**) represents separation of treatments as (1) Control, (2) *Rhizobium* inoculation, (3) GA3 (10^{-4} M), (4) GA3 (10^{-5} M), (5) *Rhizobium* inoculation + GA3 (10^{-4} M), (6) *Rhizobium* inoculation + GA3 (10^{-5} M). The abbreviations of loading plots (**B**) are as Grain Yield (GY), Stover Yield (SY), Chlorophyll contents (Chl), Number of pods (N pod), Seed carbohydrate contents (SC), Plant height (PH), Leghaemoglobin contents (Leg HB), Dry pod Weight (DPW), Protein contents (Protein), Weight of nodules (Wt Nod), dry weight of nodules (DWN), Stover K (STK), Seed N (SDN), Stover P (STP), Seed P (SDP), Stover N (STN), and Seed K (SDK).

4. Discussion

Biological nitrogen fixation (BNF) as an important source of nitrogen (N) is key process to modern sustainable agriculture. Enduring sustainability in agriculture can be achieved

by using effective management of resources. It is irrational to consider agricultural sustainability on a broader scale without biological nitrogen fixation. Plant growth promoting rhizobacteria (PGPR) are important to plant growth through producing phytohormones, fixing N_2 symbiotically, inorganic phosphate solubilizing, organic phosphate mineralization, and nutrients supplementation [50–54]. We found higher growth and yield attributes of chickpea under the application of *Rhizobium* and gibberellic acid GA_3 in a pot trial (Figures 1 and 2).

The beneficial effects of *Rhizobium* alone and supplemented with other growth stimulators in the present study are in accordance with the previous reports [55–57]. Application of plant growth regulators (PGRs) especially GA_3 exert stimulatory and beneficial effects on plant growth and development which is well studied in green gram, mungbean, chickpea, tomato, cowpea, and china aster [58,59]

The results of this study confirm the alteration in plant height in response to foliar application of GA_3 (Figure 1). In chickpea, shoot length significantly increases by inoculation with *Rhizobium* [51,52,58]. Likewise, the application of GA_3 has a positive effect on shoot and root length in chickpea [60]. It might be due to the role of gibberellins in cell multiplication, elongation, and metabolic pathways of protein synthesis. It has been observed that plants have potential to store excess of exogenously applied hormones as reversible conjugates and are released during the growth period as active hormone where and when plants need them. Earlier, it has been found that PGRs play important role in source and sink dynamics of field crops through improving distribution and transportation of accumulates [29,61].

In the present study, yield parameters were also increased significantly with foliar application of plant growth regulator inoculated with rhizobia. The foliar application of PGRs improves inflorescence, pod development, seed index, harvest index, biomass, and yield in chickpea [62]. Neelima et al. [63] observed that yield components of chickpea i.e., flower retention, number of pods, pod setting, and seed index increase significantly by foliar application of GA_3 and Cycocel®. In the present study, results depicted that nodulation is improved in *Rhizobium* treated chickpea alone and complemented with exogenous application of GA_3. This may be attributed to increased N-fixation by *Rhizobium* due to the increasing contents of nitrogenase [2]. This premise is supported by the increased accumulation of N in the plant biomass (Figure 3).

The longevity and efficacy of root nodules are favorably influenced by the exogenous application of PGRs [64]. In the present study, an increasing trend in nodule dry weight was observed after inoculation with *Rhizobium* alone or in combination with PGR. Also, *Rhizobium* inoculation supplemented with GA_3 had more positive effect and produced the maximum pods and straw yield than only *Rhizobium* inoculated plants. GA_3 is known to increase number of pods, pods yield per plant (fresh and dry) and seed index in chickpea, green gram and cow pea [14,59,65], whereas chickpea sown in soil treated with *Rhizobium* produced pods with more seeds [66]. In present study, foliar application of GA_3 on *Rhizobium* inoculated chickpea also resulted in increased chlorophyll contents [14]. These results are further substantiated by the findings of Tripathi et al. [67].

In the present study, protein contents increased with the alone or combined application of *Rhizobium* and GA_3 (Figure 2). Earlier, it has been found that exogenous GA_3 spray promote protein contents of tomato [68]. Protein content of seed is improved through application of *Rhizobium* in the present study (Figure 2). These results are in line with Rabbani et al. [55] who reported increased biochemical constituents in pea seeds. Jain et al. [69] reported the response of growth regulators (GA_3, IAA, and Kinetin) on leghaemoglobin synthesis and noted that about all PGRs expressed overall stimulatory effect on the production of leghaemoglobin. Nitrogenase activity of root nodules is raised by the increasing volume of pink bacteroid tissue. The application of GA_3 resulted in enhanced nodule bacteroid regions and leghaemoglobin contents over untreated control.

In this study, carbohydrate contents of chickpea seeds were enhanced with the combined application of GA_3 and *Rhizobium*. Under drought, the amylase activity of cotyledons is

affected severely which is significantly improved with the alone application GA_3 [70]. The application of GA_3 and rhizobium is a win-win strategy to enhance agricultural production.

In the current study, an increasing trend in the nutrient contents of grain, stover, and soil was observed. These findings are supported by Gupta et al. [71] who observed increased contents of N, P and P and higher fertilizer use efficiency under the application of PGRs. This attribute may be due to enhanced root growth and higher nodulation observed with the combined application GA_3 and *Rhizobium* in the present study. Moreover, the higher nutrient concentrations in chickpea seeds in the present study may also be due to the increased supply of nutrients due to applied rhizobia and GA_3 [72,73].

Rhizobia are well recognized plant growth promoting bacteria that enhance plant growth through plenty of mechanisms, such as nutrient solubilization, production of secondary metabolites, enhanced nitrogen fixation, and production of plant growth hormones [12,15]. The application of gibberellic acid on the other hand enhances plant growth by mediating of root nodulation process in legumes through cross talk with cytokinins signaling pathways [28]. This synergy was observed in the present study, evidenced by the enhanced root nodulation under the application of gibberellic acid and rhizobium in combination. Moreover, the applied rhizobia have shown the capacity to synthesize gibberellic acids and IAA along with other growth promoting activities (Table 1), which might have contributed to enhanced growth, yield, and nutritional quality parameters of chickpea.

5. Conclusions

The present study clearly showed that the combined application of *Rhizobium* and gibberellic acid (GA_3) played a significant role in improving the growth, yield, physiological, and biochemical attributes of chickpea. It is worthy to mention that by comparing the independent application of both, the combined application showed more promising results. The combined use of *Rhizobium* and plant growth regulator would be an effective approach to enhance legume productivity. However, multi-sites field trials are required to warrant the successful performance of the technology under natural field conditions.

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