Crack-entry invasion of wheat roots by *Azospirillumbrasilensevia* chemical-enzyme treatment: a way facilitating para-nodule formation and forced association for proper crop yield

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Abstract— *The induction of nodule-like structures referred to para-nodules was assessed due to 2,4-D, IAA and the enzyme mixture of cellulase and pectolyase in presence of PEG treatment of wheat cultivars inoculated with Azospirillumbrasilense. In gnotobiotic model experiment; 9-18 and 6-19 para-nodule plant^{-1} were produced due to 2,4 D and IAA treatments respectively. Less than 7 para-nodules plant^{-1}were attributed to Azospirllum alone, numbers increased to 26 when the diazotroph was introduced in combination with p-nodule-inducing agents. The cell wall-degrading enzyme mixture with PEG facilitated the crack-entry invasion of the diazotroph in population of $> 5x10^5$cfu g^{-1} root. High rates of C$_2$H$_2$ reductions of > 200 nmoles C$_2$H$_4$ g^{-1} root hr^{-1} were estimated for the enzyme mixture-PEG treated plants. The average glutamine synthetase activities of plant leaves were the highest (57.1-86.3 µ mol g^{-1}Fw hr^{-1}) for IAA-Azospirillum treatment. Wheat plants successfully paranodulated in pot experiment when pre-treated with IAA and enzyme mixture and inoculated with Azospirillum in presence of 50 % of recommended N, plant biomass and N yields increased as well. The highest levels of chlorophyll a (2.10 µg g^{-1}Dw), chlorophyll b (2.28) and carotenoids (1.59) were estimated for inoculated plants pre-treated with IAA plus enzyme mixture. In the field trial, as high as > 2.0 kg plot^{-1} total biological yield was produced by plants initially primed by soaking in water. The superior grain yields of 441.7-571.4 g plot^{-1} were attributed to Azospirillum inoculation together with IAA and enzyme mixture for 50 % N-supplied plants. Seed priming somewhat raised the grain yield.*

I. INTRODUCTION

In 1989, the term of para-nodule was introduced by [1] to describe chemically and/or enzymatically induced nodule-like knots or outgrowths formed on non-legume root systems. These special structures are differed from those known to naturally produced by rhizobia-inoculated leguminous plants. Among the chemical compounds that support the production of nodule-like root tumours; 2,4-D (2,4-dichloro-phenoxyacetate), IAA (indole acetic acid) and tryptophan are the universally used [2-4]. This opened a window for microbiologists to search for the possible contribution of these nodular structures in the plant-microbiota interweave and consequently to crop production. Among the strategies adopted to magnify the benefits from N$_2$-fixation by diazotrophs to cereal crops, in particular, are to induce the formation of tumour-like outgrowths by the use of the auxin 2,4-D which successfully created a large number of para-nodules on wheat plants [5]. When introduced into the plant-soil system, diazotrophs occupy the root theater and penetrate the paranodules by migrating in between loosely arranged cells which covered their outer layers or *via* invading the spaces at the junction of root and para-nodules. When wheat plants were treated with 2,4-D and inoculated with the cyanobacterium*Nostoc* sp. and kept to grow in N-deficient medium, the estimated ethylene production levels were 3-folds compared to the auxin-untreated plants although the root system largely colonized by the cyanobacterium. In an attempt to study the effect of *Azospirillumbrasilense* inoculation on para-nodule

formation on maize roots, [6] found that the bacterium succeeded to produce the root-tumours and existed inside recording appreciable levels of C_2H_2 reduction. When determined by $^{15}N_2$ enrichment method, the nitrogen fixed by the NH_4^+-excreting diazotroph was transferred from the bacterium to the host plant and significantly increased in para-nodules. [7]indicated C_2H_2 reduction in 2,4-D treated or untreated wheat plants in presence of *Azotobacter* and *Beijerinckia*. But the authors failed to force an association between *A. paspali* and *Paspalumdilatatum* in presence of 2,4-D.

Apart from out-growths formation due to chemical treatment, [8] reported the induction of nodular structures on rice roots when young seedlings of only 2 days were treated with the cell wall degrading enzyme mixture of cellulase and pectolyase in presence of polyethylene glycol (PEG) and rhizobia inoculation. The authors mentioned that although acetylene reducing activities of the developed outgrowths were in the sensitivity levels of the assay procedure, their work is the first on the formation of root nodules on a non-legume due to rhizobia inoculation. Such a phenomenon paved the way on the possible nodulation of plant varieties other than legumes.

In fact, no much work has been done on the effect of simultaneous chemical and enzymatic treatment on wheat when inoculated with N_2-fixing bacteria. Therefore, this work was planned to throw some light on the combined chemical-enzyme treatment effects on wheat-*Azospirillum* interweave to force an endophytic association under gnotobiotic, greenhouse and field conditions.

II.   MATERIALS AND METHODS
Wheat cultivars

Three high yielding wheat cultivars; Sakha 94, Giza 168 and Gemeza 10, kindly provided by Field Crops Institute, Agricultural Research Center, Giza were the plant materials. Initially, seeds were tested for germination rate on moistened cotton in Petri dishes; after 5 days, a germination percentage of >95 percent was scored.

Diazotroph strain

An endophytic *Azospirillumbrasilense*s train isolated by [9] was used. The bacterium is characterized by N_2-fixation efficiency of 66.9 mg N fixed /g C consumed and production of indole acetic acid (IAA), gibberellins and cytokinins in quantities of 59.4, 9.3 and 313 ppm respectively. The diazotroh was cultured to late log phase in N-deficient synthetic malate medium of [10]. The medium contains (g^{-1}): Dl-malic acid (5.0), KOH (4.5), KH_2PO_4 (0.6), K_2HPO_4 (0.4), $MgSO_4.7H_2O$ (0.2), NaCl (0.1),

$CaCl_2.2H_2O$ (0.02), $MnSO_4.4H_2O$ (0.01), $Na_2MoO_4.2H_2O$ (0.002), Fe (III)-EDTA (0.66 % [wt/vol] in water, 10 ml); biotin (0.1 mg), NH_4Cl (0.5), yeast extract (0.1). For inoculation, seeds or roots of 2-day old seedlings were immersed for 30 min. in a freshly prepared diazotroph suspension of *ca*. 10^8 cells ml^{-1}.

Gnotobiotic model experiment

Chemical and enzyme treatments for 2-day old wheat seedlings in presence of *Azospirillumbrasilense*for para-nodule production and crack-entry invasion by the diazotroph were applied in gnotobiotic model experiment.

Seeds of cvs.Sakha 94, Giza 168 and Gemeza 10,were surface sterilized by immersion in 0.1 % (w/v) mercuric chloride for 10 min. then washed thoroughly with sterile distilled water, this was followed by germination on water agar in dark at ambient temperature. Roots of 2-day old seedlings were treated with the enzyme mixture of 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase in 6.0 % (w/v) mannitol. This was done for 10 min. @ 22 °C. Following the enzyme mixture treatment, seedling roots were dipped for 30 min. in 20 ml of autoclaved aqueous 20 % (w/v) polyethylene glycol (PEG) containing 50 mµ $CaCl_2$.Seedlings either inoculated with*Azospirillumbrasilense* or not were transferred into glass tubes (6 cm diameter and 40 cm length) one third filled with carbon-and nitrogen-lacking agar medium of [10]. Such growth medium was supplied with either 2,4-dichloro-phenoxyacetate (2,4-D) or IAA in concentrations of 2.0 or 4.0 ppm respectively.

Seedlings of the different chemical, enzymatic and inoculation treatments were kept for 45 days @ ambient temperature. Thereafter, plants were gently taken and developed para-nodules were counted and examined by naked eyes and low-magnification microscopy after staining with 2,3,5-triphenyltetrazolium chloride [11]. For enumeration of *Azospirillum*inside the root tissues, the entire root system of inoculated seedlings was separated, sterilized with isopropanol (1 min.) then in 3.0 % sodium hypochloride for 1.5 hours followed by 3 washes with sterilized distilled water. After surface sterilization, roots were crushed with adequate volume of basal nutrients of N-free malate medium and decimal serial dilutions were prepared for counting the endophyte using plate count technique.

For acetylene reduction assay, plant roots of inoculated treatments were aseptically transferred to test tubes and sealed with rubber closers. Ten percent of the gas phase was replaced by acetylene, tubes were incubated for 24 hr. @ 28 °C. Half ml of the gas mixturewasinjected into

gas chromatograph and ethylene produced was measured [12].

The glutamine synthetase (GS) activity was measured in leaves of para-nodulated plants adopting the spectrophotometric procedure described by [13]. Plant roots and shoots were determined for dry weights (70 °C to constant weights) and nitrogen contents [14].

Greenhouse experiment

The most responsive two wheat cultivars (Giza 168 and Sakha 94), among the three tested ones in gnotobiotic experiment, to chemical and enzyme treatments were examined for forced association with *Azospirillum* when the diazotroph was introduced together with IAA and the enzyme mixture of cellulase and pectolyase. A fertile clay loam soil (organic carbon, 0.68 %; total nitrogen 0.099 %, EC, 2.3 dSm^{-1}; pH, 7.9) collected from the Experimental Station of Faculty of Agriculture, Cairo University, Giza was distributed in plastic pots (20 cm diameter, 25 cm depth) @ the rate of 5 kg pot^{-1}. The recommended PK fertilization regimes equivalent to 150 and 50 kg acre^{-1} of superphosphate (P$_2$O$_5$, 15.5 %) and potassium sulphate (K$_2$O, 50 %) were incorporated into soil and thoroughly mixed prior to planting. Ammonium sulphate (20.6 % N) @ full rate of 50 kg N acre^{-1} and/or half rate were incorporated into soil as well. At the rate of 20 pot^{-1}, seeds of wheat cultivars were sown. Seeds were treated with IAA, enzyme mixture or *Azospirillum* as mentioned in gnotobiotic model experiment. Pots were arranged in the greenhouse in a complete randomized design with three replicates. At harvest (120 days), plants were gently uprooted without tearing of root system as possible and assessed for para-nodule production, dry weight (70 °C to constant weight), chlorophyll a, b and carotenoids [15]. Adopting the method of [14], nitrogen contents for plants were determined.

Field trail

The superior bio-chemical treatment in respect to para-nodule formation and wheat growth was experimented using Sakha 94 and Giza 168 cultivars with and without seed priming. Initially, the traditional agricultural practices recommended for wheat cultivation, soil ploughing and compacting were applied. The experimental area was divided into plots of 2m^2 (2m x 1m) including 5 rows, each treatment was replicated 4 times in a complete randomized block design. Superphosphate (P$_2$O$_5$, 15.5 %) and potassium sulphate (K$_2$O, 50 %) were incorporated into soil at the rates equivalent to 150 and 50 kg acre^{-1} during land preparation. N fertilization in the form of ammonium sulphate (N, 20.6 %) was add at either full recommended dose of 50 kg N acre^{-1} or its half depending upon treatment in two splits,

after 40 and 70 days of sward establishment. Prior to seed treatment with IAA, enzyme mixture or *Azospirillum* as previously mentioned, seeds were soaked in water for 24 hours [16] following drying just before radial emerges.

The experimental layout comprised 5 treatments for either water-primed or -unprimed seeds. Those are: 1) untreated (control), 2) 100 % N fertilizer, 3) 50 % N fertilizer, 4) 50 % N fertilizer + *Azospirillum* and 5) 50 % N fertilizer + IAA+Enzyme + *Azospirillum*. At harvest, plants were collected and determined for para-nodulation as well as total biological, grain and protein yields.

Data analysis

All the experimental data are means of three or four replicates and the treatment means were compared at the confidence level of 5 % [17]. Liner regressions and correlation coefficients among a number of traits were considered as well.

III. RESULTS

The untreated 45-day old wheat seedlings developed in gnotobiotic model experiment hardly para-nodulated (Table 1). Treatment with the auxin 2,4-D consistently induced nodule-like structures that appeared round in shape, 9-18 *p*-nodules per plant were produced being the highest on the root system of cv. Sakha 94. Similarly, IAA stimulated the induction of such nodular outgrows (6-19 plant^{-1}) being the lowest with cv. Gemeza 10. Treatment with the cellulase-pectolyase enzyme mixture together with polyethylene glycol(PEG) occupied a medium rank among the tested *p*-nodule-inducing agents. *Azospirillumbrasilense* alone was not that successful for the cereal paranodulation (< 7 *p*-nodule plant^{-1}, in average). The diazotroph actively acted when introduced in combination with any of the other tested materials where higher numbers of tumor-knots (up to 26 plant^{-1}) were formed along the primary roots. Apart from bio-and chemical-treatments, cv. Sakha 94 overcame other cultivars in para-nodulation recording the higher number of 14 plant^{-1} in average followed by cv. Giza 168 (13) and cv. Gemeza 10 (9).

The cell wall-degrading cellulase and pectolyase enzyme mixture with PEG treatment supported the highest endophytic establishment of the diazotroph where numbers of > 5x10^5 cfu g^{-1} root were scored (Fig.1). No obvious differences were obtained among the auxins 2,4-D and IAA, respective average numbers of 1.2 x 10^4 and 1.7 x 10^4 cfu g^{-1} root were estimated. The invasion rates of *Azospirillum* to the inner tissues of the cereal plant were the lowest when the bacterium was introduced alone (<10^4 cfu g^{-1} root). The enzyme-PEG treatment did facilitate more entry of the

bacterium into root system. Irrespective of treatment, the endorhizosphere of wheat cv. Giza 168 accommodated the highest diaztroph population (14.5 x 10^4 g^{-1} root, in average) with cv. Gemeza 10 being the inferior (< 6 x10^4 g^{-1} root).

Acetylene reducing activity variably detected for the different *Azospirillum* inoculation treatments (Fig. 2). The highest estimates of nitrogenase activities (83 -> 200 nmoles C_2H_4 g^{-1} root hr^{-1}) were scored for the enzyme mixture-PEG treated plants. Other para-nodule inducing agents somewhat promoted ethylene production over the diazotroph alone.

In absence of *Azospirillum*, the IAA-treated plants produced significantly higher root biomass compared to untreated correspondings, this was not the case for 2,4-D-treated ones (Table 1). No stimulatory effect was attributed to the enzyme-PEG treatment. For the diazotroph-inoculated treatments, the auxins applied intensified the root biomass production, cv. Sakha 94 responded much better than other cultivars. Fluctuations in shoot dry matter yields among the experimental treatments behaved in an akin trend to that recorded with root dry weights.

Interestingly, in the majority of cases, the auxin treatment particularly in the presence of *Azospirillum* modified the root: shoot ratio, an observation scored with the three tested wheat cultivars.

Little quantities of nitrogen were accumulated in the root systems of wheat particularly the untreated ones (Table 1). For uninoculated plants, IAA seemed more favourable for N accumulation than 2,4-D. Significantly higher amounts of N were present in root systems when the diazotroph was introduced. Compared to root N pool, areal parts of the cereal contained more N, this was recorded for all the examined cultivars. Again, *Azospirillum* particularly in the presence of *p*-nodule inducing chemicals supported higher N levels in plant shoots.

Relatively low glutamine synthetase enzyme activities were attributed to *p*-nodule inducing agents in leaves of uninoculated plants, estimates are falling in the range 48.3-59.1 μ mol g^{-1}Fw h^{-1} depending upon the material and cultivar (Fig. 3). *Azospirillum* inoculation of chemical-treated plants raised the enzyme activity up to > 78 %. The average enzyme activities of the chemical-diazotroph treatments could be arranged in the descending order: IAA (74.0 μ mol g^{-1}Fw h^{-1}) > 2,4-D (69.7) > enzyme-PEG (67.0). Irrespective of treatments, root systems of cv. Sakha 94 exhibited the highest average glutamine synthetase activity (66.4 μ mol g^{-1}Fw h^{-1}) followed by cv. Giza 168 (63.7) and cv. Gemeza 10 (54.5).

The successful paranodule-inducing auxin IAA and the cell wall degrading enzyme complex of cellulase and pectolyase were further studied in a pot experiment for para-nodulation and wheat growth in presence of *Azospirillum* and rational N fertilization. Themost responsive wheat cultivars
in gnotobiotic model experiment, *i.e.*, Sakha 94 and Giza 168 were the plants tested.

The heavy N-dressed plants rarely para-nodulated, this was observed with both cultivars (Table 2). On the contrary, those rationally-supplied with N and received either IAA, enzyme mixture or *Azospirillum* produced high numbers of nodular outgrowths with large sizes. Roots of plants inoculated with the diazotroph simultaneously pre-treated with IAA and enzyme mixture hosted 23-34para-nodules $plant^{-1}$.Generally speaking, cv. Sakha 94 nodulated much better (average of 15 $plant^{-1}$) than cv. Giza 168 (11 $plant^{-1}$).

Full N-fertilizerdcvs.Sakha 94 and Giza 168 produced 133.1 and 96.5 % higher plant biomass yields compared to those left with no N fertilizer. When supplied with half dose of recommended N and pre-treated with IAA and enzyme mixture, dry matter yield slightly increased by 1.6 and 5.0 % respectively. *Azospirillum* inoculation seemed unavoidable to promote biomass yield production. The diazotroph in combination with 50 % of recommended N level produced yields comparable to those of full N-supplied plants. Increases in biomass yields of 16.3 and 9.0 % were estimated for cvs. Sakha 94 and Giza 168 respectively when plants were pre-treated with the auxin and enzyme mixture.

As expected, the full-N supplied plants contained the highest N levels (44.09 mg $plant^{-1}$ in average). Reducing the N input to 50 % lowered the plant N pool even in the presence of the N_2-fixing bacterium in the plant-soil system. The stimulatory influence of the diazotroph on wheat N yield was magnified when seeds were pre-treated with IAA and the enzyme mixture where yield of*ca.*40 mg $plant^{-1}$,somewhat comparable to full N-fertilized plants, was obtained. In absence of inoculation, the effect of IAA and enzyme mixture together was not that successful for N accumulation where average content of 22.84 mg $plant^{-1}$was scored.

Ear dry weights significantly affected by the applied treatments, being the lowest for untreated plants (272.5 mg $plant^{-1}$ in average). Plants pre-treated with IAA plus enzyme mixture simultaneously inoculated with *Azospirillum* produced the highest ear dry weights of 692 and 666 mg $plant^{-1}$ for cvs. Sakha 94 and Giza 168 respectively.

Nitrogenfertilization and diazotroph inoculation significantly increased chlorophylls a, b and carotenoids over untreated plants. Apart from wheat cultivar, the average superior levels of chlorophyll a (2.10 μg g^{-1} Dw), chlorophyll b (2.28) and carotenoids (1.59) were scored for IAA-enzyme mixture pre-treated plants in combination with diazotroph inoculation.

To what extent the cereal seed priming with water prior to treatment and cultivation might accelerate growth and increase yield? A question answered in the field trial. Table (3) shows that as high as 33 nodule-like knots were formed on root system of primed cv. Giza 168 pretreated with IAA and enzyme mixture in presence of *Azospirillum*. On the contrary, Sakha 94 plants either untreated or full N dressed failed to para-nodulate. Irrespective of cultivar and treatment, *p*-nodules produced by unprimed plants represented *ca*. 80 % of those formed by the primed correspondings.

The rational N fertilizer regime intensified the promoting influence of diazotroph inoculation particularly when plants were pre-treated with the auxin and enzyme complex, a phenomenon noticed with both cultivars. In such treatment, the highest total biological yields of 2.65 and 2.36 kg plot^{-1} in average were scored for cvs. Sakha 94 and Giza 168 respectively. In general, the average total biological yield produced by primed plant was 2.01 kg plot^{-1} against 1.92 kg plot^{-1} for those left without seed priming. Again, *Azospirillum* inoculation of wheat pre-treated with the auxin and cell wall degrading enzyme mixture accumulated 9.0 % more protein compared to full N supplied ones, protein content of primed plants exceeded, in average, those unprimed by 2.67 kg pot^{-1}.

Untreated plants produced 323.2-386.1 g grains plot^{-1}. Full N supplementation raised the grain yield up to 488.8 g plot^{-1}. Reductions of 5.95-19.54 % were attributed to reducing the fertilization rate to 50 %. Diazotroph inoculation particularly in presence of other pre-treatments did conspicuously compensate these reductions. Here, the superior grain yields of 441.7-571.4 g plot^{-1} were obtained by inoculated plants together with IAA and enzyme complex. Seed priming, apart from other treatments, significantly supported higher grain production, all of all average increase of 2.24 % over unprimed plants was estimated.

IV. DISCUSSION

Recently, great efforts are being done to create artificial and forced atmospheric dinitrogen-fixing symbiotic association with non-leguminous plants that are unable to establish such close interaction in a natural way. This is a promising approach to magnify the efficiency of N$_2$ fixation of associative diazotrophs with non-legumes. In these very special endosymbiosis, not only rhizobia are the key players, but associative microsymbionts may have a prominent contribution. The last few decades intensed several reports dealing with induction of new growth structures referred to as paranodules that could be formed on the root systems of several non-legume plants such as rice, barley, wheat and rape seed [2,4,18,19]. As reported by [3], the N$_2$-fixing appeared to occupy the cracks surrounding the sites of root emergence in wheat beside the others associated with the nodular structures induced after treatment with the auxin 2,4-D. Strikingly, the colonization rate of the N$_2$-fixing candidate was more pronounced on the chemically induced para-nodules compared to the correspondings on the lateral root emergence sites that were not treated with the chemical compound.

Besides the 2,4-D-paranodule inducing auxin; IAA in presence of *Azsospirillum* were experimented in the present study for para-nodule formation on a number of wheat cultivars. Forced endophytic association between the cereal plant with the diazotroph*via* chemical and enzyme treatments were among the target of the present study. Naked eye observations showed no uniformal distribution of nodular structures along the whole root systems, where the majority were located at the tip of the root. Both 2,4-D and IAA successfully induced the outgrowths on roots of the tested wheat cultivars (up to 19 plant^{-1}). *Azospirillum*, in absence of nodular-inducing agents, resulted in < 7 *p*-nodules plant^{-1}. The cultivar effect was very obvious where cv. Sakha 94 hosted as high as 14 *p*-nodules plant^{-1} against 9 plant^{-1} for Gemeza 10. Light microscopic examinations of the tip appeared star-shaped owing to the nodular structures that emanate from a central root where *Azospirillum* colonized the spaces between the induced outgrowths.

Several genera of associative diazotrophs have been examined for their ability to penetrate and reside inside nodule-like structures of a variety of host plants and perform atmospheric dinitrogen fixation. Results of [5] indicated that N$_2$-ase activities of *Nostoc* sp. colonizing the wheat para-nodules substantially differ from those reported for other diaztrophic members. For instance, N$_2$-ase activities estimated for diazotrophs associated with wheat outgrowths were falling in the range from zero [20] to 40 nmoles C$_2$H$_4$ h^{-1} plant^{-1}[21]. In the present study, ethylene production rates per wheat root reached > 200 nmoles C$_2$H$_4$ g^{-1} h^{-1}. Such extraordinary levels of acetylene reducing activities is most probably attributed to the high N$_2$-fixation

efficiency (66.9 mg N fixed/g C consumed) of the used *Azospirillumbrasilense* strain. Here, it has to be mentioned that in forced associations of diazotrophs and plant roots, it appears that the O_2 tension of the environment seemed prominent in the activity of the microorganism to fix N_2. [18]reported that even though 2,4-D supports higher tolerance of *Azospirillum* towards oxygen, acetylene reducing activity was considerably low at O_2 level of > 5 k Pa [22]. [5]explained why treated-para-nodulated roots exhibited higher acetylene reducing activities than those kept untreated, that induced para-nodules provide additional sites for the diazotroph to colonize and fixes N_2.

Studies of[19] indicated the formation of nodular structures on wheat roots treated with 2,4-D or inoculated with a N_2-fixing composite formulation including *Arthrobacter* sp. and *Xanthomonas* sp. Scanning electron microscopic examinations of these structures revealed surface and internal colonization beside the formation of the para-nodules. The authors believed that this mucigel most probably create a microenvironment suitable for the introduced diazotroph to penetrate into root tissues, colonize and fix N_2. This mucous material certainly restricts the O_2 diffusion to the para-nodules and diazotrophs, this improves the activity of the very O_2-senstive nitrogenase enzyme.

It is worth to mention that, nitrogenase activities by the used *Azospirillumbrasilense*in this study were comparatively higher in all IAA, 2,4-D and Enz-PEG treatments than the diazotroph-free controls. These findings are contradicting those of other investigators [23, 24] using other N_2-fixing microbiota such as *Azotobactervinelandii* where the auxin 2,4-D poorly supported biological nitrogen fixation. They attributed such low levels of N_2-fixation to the deficiency in the tumor-cell infection. In addition, [25] found that *Azorhizobiumcaulinolans* was occasionally localized inside epidermal and cortical tumor cells, with no bacterial infection, either inter-or intra-cellular, to inner para-nodule tissues. This might explain why N_2-fixation was not improved in case of 2,4-D treatment.

Wheat development in terms of biomass and nitrogen yields markedly improved by the *p*-nodule inducing agents, an effect that intensified by *Azospirillum*inoculation. Effects, in most cases, reached the levels of significance. These results are in conformity of those of [26] who reported significant increases in nitrogenase activity, wheat biomass and grain yields in the descending order: 2,4-D together with *Azospirillum*> the diazotroph alone > either 2,4-D or control. In accordance, as well, are the findings of [27] who reported great promotion

for wheat growth by 2,4-D and IAA addition in presence of the plant growth promoting *Azotobacterchrococcum* and *Pantoeaagglomerans.*On the other hand and contradicting the findings of the present study, [19] found that when wheat plants were treated with the diazotroph*B. polymyxa* 43 alone or a mixed culture of *Arthrobacter* sp. and *Xanthomonas* sp. with 2,4-D, the cereal biomass yield decreased.

Total chlorophyll as well as carotenoid pools of para-nodulated wheat cultivars obviously increased for all auxin-and enzyme-treated wheat, an observation that recorded only in presence of *Azospirillum.* This was not the case in absence of the diazotroph. Similar results were reported by [4] for *Cymbopagonwinterianus*para-nodulated by 2,4-D and *Azospirillumbrasilense*.

Results of the pot experiment, as a model, indicated that paranodulation of wheat plants positively correlated with both biomass yield (R= 0.949) and N content (R= 0.983). This is expressed in the linear regressions illustrated in Figure (4). The calculated correlation matrix (R-values) for the interactions between the various determined growth parameters is presented in Table (4).

In 2013, [16] recommended seed priming by soaking in either water or in a solution of specific salts for a specified period of time. This should be followed by air-drying in shade, such a primitive treatment is necessary to be done immediately prior to radial emergence. The authors emphasized that seed priming improves faster and synchronized germination not only in stressed environments but under natural conditions. The design of the field trial executed in the present work comprised, beside N fertilization and *Azospirillum* inoculation with IAA and enzyme mixture, priming of wheat seeds by soaking in water for 24 hours. In addition to the stimulatory effects of chemical and biological treatments on plant development (Fig. 5), seed priming positively acted to

support better grain yield (Table 5). It is of rather interest, seed priming was found to be cultivar-dependent, where wheat cv. Giza 168 highly responded to water soaking compared to cv. Sakha 94. Increases, over unprimed plants, in grain yields ranged from 0.7 to 20.5 % for the former cultivar and 0.8 to 5.7 % for the latter.

In conclusion, the para-nodules induced by either IAA, 2,4-D or Enz-PEG treatments might have served as niches for diazotrophs colonization of non-legume plant roots thereby higher atmospheric dinitrogen fixing capacity in root tissues. This conspicuously reflects on crop productivity and human welfare.

REFERENCES

[1] Tchan YT and Kennedy IR. 1989. Possible N₂-fixing root nodule induced in non-legumes. *Agric. Sci. (AIAS, Melbourne)* **2**: 57-59.

[2] Youssef H, Monib M, Fayez, M and Hegazi NA. 1998. Induction of paranodulation by 2,4-D, IAA and tryptophan in wheat inoculated with various diazotrophs. In: Nitrogen Fixation with Non-Legumes. (Malik, K.A., Mirza, M.S. and Ladha, J.K., eds). *Kluwer Academic Publishers*, pp: 145-150.

[3] Pereg-Gerk I, Sar N and Yaakov L. 2002. In situ nitrogen fixation associated with sea grasses in the Gulf of Elate (Red Sea). *Aquat. Ecol.*, **36**:387-394.

[4] Saikia SP, Goswami A, Mudoi KD, Gogi A, Kotoky R, Lekhak H and Handique, N. 2014. Effect of 2,4-D treatment and *Azospirillum* inoculation on growth of *Cymbopogonwinterianus*. *Afri. J. Microbiol. Res.*, **8(9)**:955-960.

[5] Gantar M and Elhai J. 1999. Colonization of wheat para-nodules by the N₂-fixing cyanobacterium*Nostoc* sp. strain 2S9B. *New Phytol.*, **141**:373-379.

[6] Christiansen-Weniger C. 1993. Para-nodule induction in maize with indole-acetic acid (IAA) and its infection with ammonia excreting *Azospirillumbrasilense*. In: Hegzi, N.A.; Fayez, M. and Monib, M. (eds), Nitrogen Fixation with Non-Legumes. *The American University in Cairo Press*, pp. 525-536.

[7] Tchan YT and Zeman AMM. 1995. N₂ fixation (C₂H₂ reduction) in 2,4-dichlorophenoxyacetic acid (2,4-D) treated wheat inoculated with free-living diazotrophs. *Soil Biol. Biochem.*, **27(4-5)**: 453-457.

[8] Al-Mallah MK, Davey MR and Cocking EC. 1989.Formation of nodular structures on rice seedlings by rhizobia. *J. Expt. Bot.*, **41**: 1567-1572.

[9] Badawi H. Mona. 2014.Diazotroph strain-wheat cultivar affinity guarantees proper cereal yield and soil nitrogen budget. *Res. J. Agric. Biol. Sci.*, **10(1)**: 8-16.

[10] Reinhold B, Hurek T and Fendrik I. 1985. Strain-specific chemotaxis of *Azospirillum* spp. *J. Bacteriol.*, **162**: 192-195.

[11] Patriquin DG and Dobereiner J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Can. J. Microbiol.*, **24**: 734-742.

[12] Hegazi NA, Amer HA and Moni, M. 1980. Studies on N₂-fixing spirilla (*Azospirillum* spp.) in Egyptian soils. *Rev. Ecol. Biol. Soil*, **17**:491-499.

[13] Mohanty B and Fletcher JS. 1980. Ammonium influence on nitrogen assimilatory enzymes and protein accumulation in suspension culture of pearl scarlet rose. Physiol. Plant, **48**:453-459.

[14] Kresovic M and Licina V. 2003. Estimation of soil nitrogen availability. *J. Agric. Sci.*, **48(1)**: 21-38.

[15] Porter H andVillar R. 1997. The fate of acquired carbon in plants: chemical composition and construction cost. Pages 39-72. In: Bazzaz, F. A. and Grace, J. editors. *Plant Resource Allocation. Academic Press, San Diego, California, USA.*

[16] Abraha B and Yohannes G. 2013. The role of seed priming in improving seedling growth of maize (*Zea maize* L.) under salt stress at field conditions. *Agric. Sci.*, **4(12)**: 666-672.

[17] Huang LS and Chen J. 2008. Analysis of Variance, Coefficient of Determination and F-Test for Local Polynomial Regression. *The Annals of Statistics*, **36(5)**:2085-2109.

[18] Yu D and Kennedy IR 1995. Nitrogenase activity (C2H2 reduction) of *Azospirillum* in 2,4-D-induced root structures of wheat. *Soil Biol. Biochem.*, **27**:459-462.

[19] Biabani A, Boggs LC, Natasha K and Morad U. 2012. Microscopic morphology of nitrogen fixing paranodules on wheat roots. *African J. Biotechnol.*, **11(12)**:2971-2976.

[20] Kennedy IR and Tchan YT. 1992. Biological nitrogen fixation in non-leguminous field crops: Recent advances. *Plant Soil*, **141**:93-118.

[21] Katupitiya S, New PB, Elmerich C and Kennedy IR. 1995. Improved N₂ fixation in 2,4-D treated wheat roots associated with *Azospirillumlipoferum*: studies of colonization using reporter gene. *Soil Biol. Biochem.*, **27**: 447-452.

[22] Christiansen-Weniger C. 1996. Endophytic establishment of *Azospirillumcaulinodans*through auxin-induced root tumors of rice (*Oryza sativa* L.).*Biol. Fertil. Soils*, **21**:293-302.

[23] Ridge RW, Ride KM and Rolfe BG. 1993. Nodule-like structures induced on the roots of rice seedlings by addition of the synthetic auxin 2,4dichloro-phenoxy-acetic acid. Aust. *J. Plant Physiol.*, **20**:705-717.

[24] Huang SZ and Jia DW. 1994. Induction of nodulation on the roots of non-legume rice. In: Nie, Y.F.; Kennedy I.R.; Chen, T.W. (eds). International Symposium on Nitrogen Fixation with Non-

Leguminous Crops. *Qingdao Ocean University Press,* pp:45-52.

[25] Christiansen-Weniger C and Vanderleyden J. 1994. Ammonium-excreting *Azospirillum* sp. become intercellularly established in maize (*Zea mays*) para-nodules. *Biol. and Fertil. Soil,***17(1)**:1-8.

[26] Elanchezhian R and Panwar JDS. 1997. Effects of 2,4-D and *Azospirillumbrasilense* on nitrogen fixation, photosynthesis and grain yield in wheat. *J. Agro. Crop. Sci.,***178**:129-133.

[27] Narula N, Deubel A, Gans W, Behl RK and Merbach W. 2006. Paranodules and colonization of wheat roots by phytohormone producing bacteria in soil. *Plant Soil Environ.,***52**: 119-129.
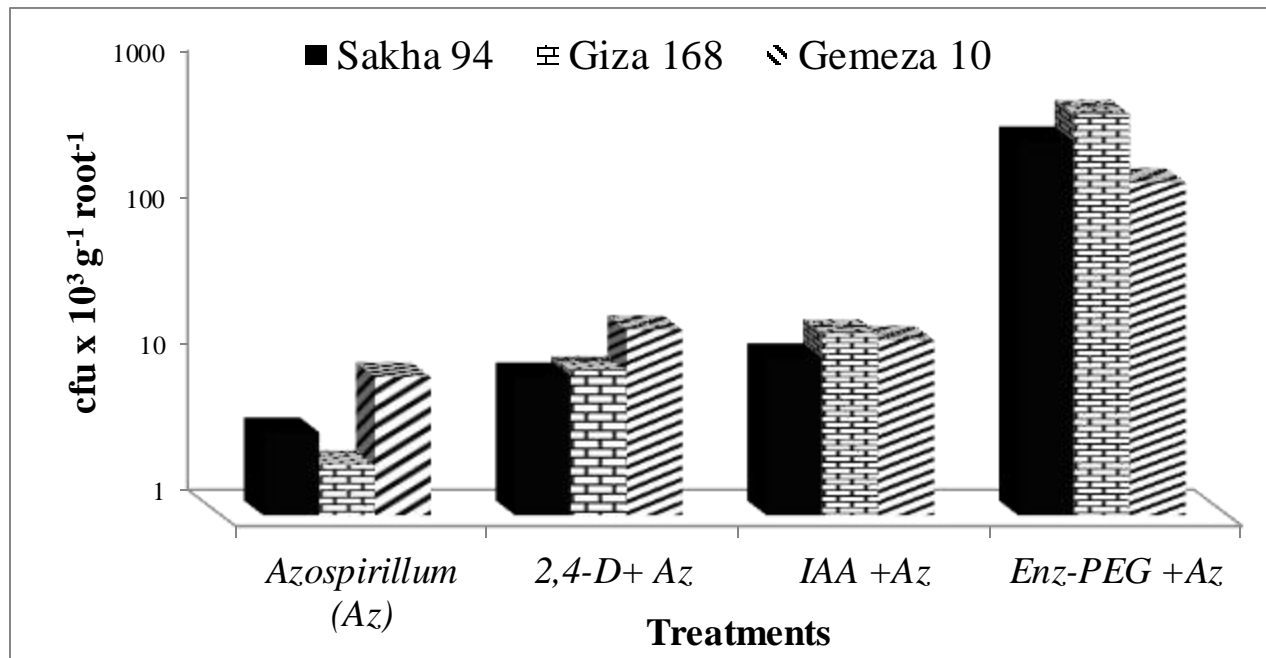
Fig. 1.Endorhizosphere populations of Azosprillum of wheat cultivars after 2,4-D, IAA and enzyme mixture-polyethylene glycol treatments.
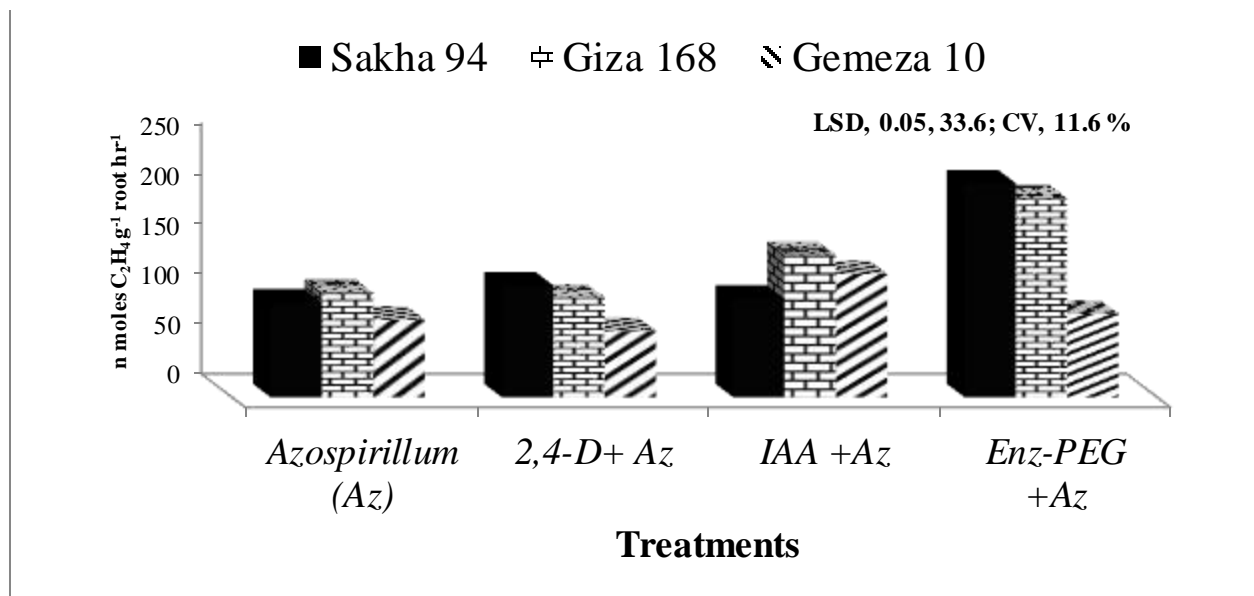
Fig. 2. Acetylene reducing activity of wheat roots of the different treatments.
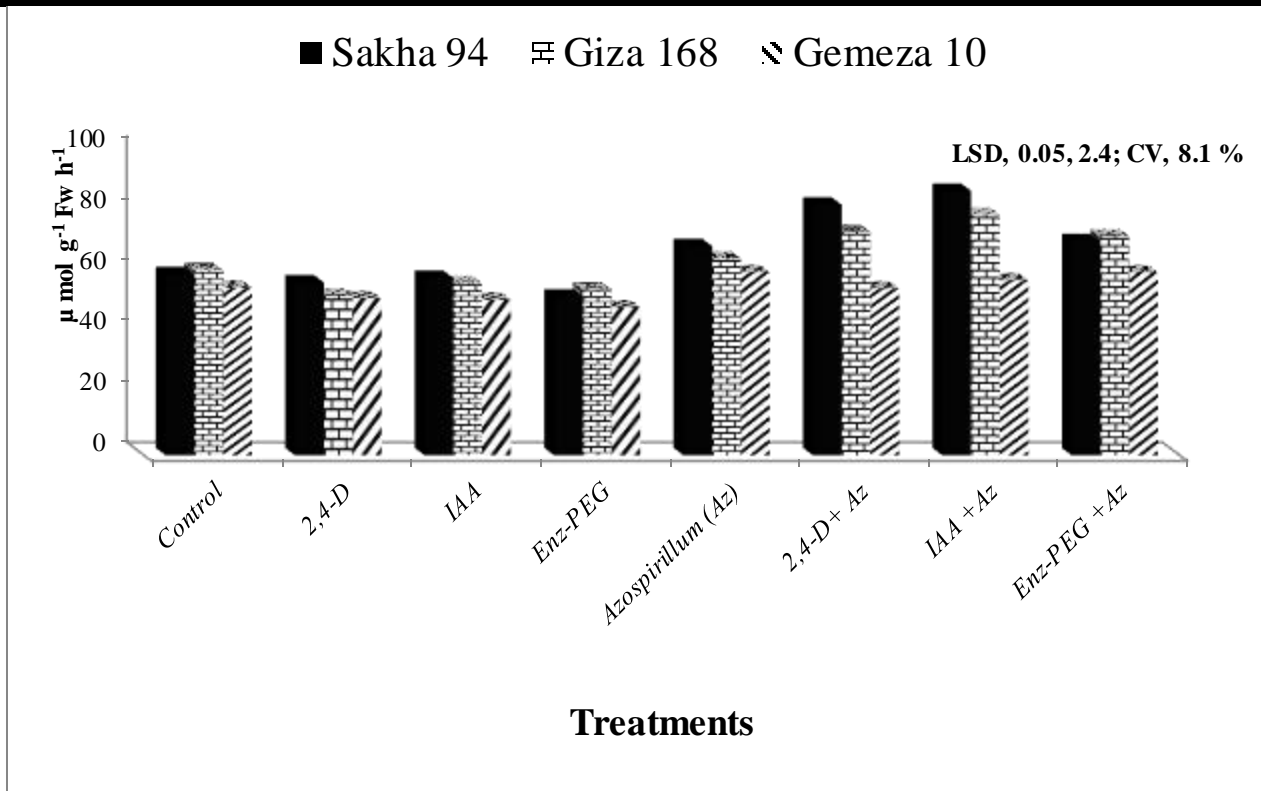
Fig. 3.Glutamine synthetase activities (μ mol g^{-1}Fw h^{-1}) of wheat leaves of the different chemical and inoculation treatments.
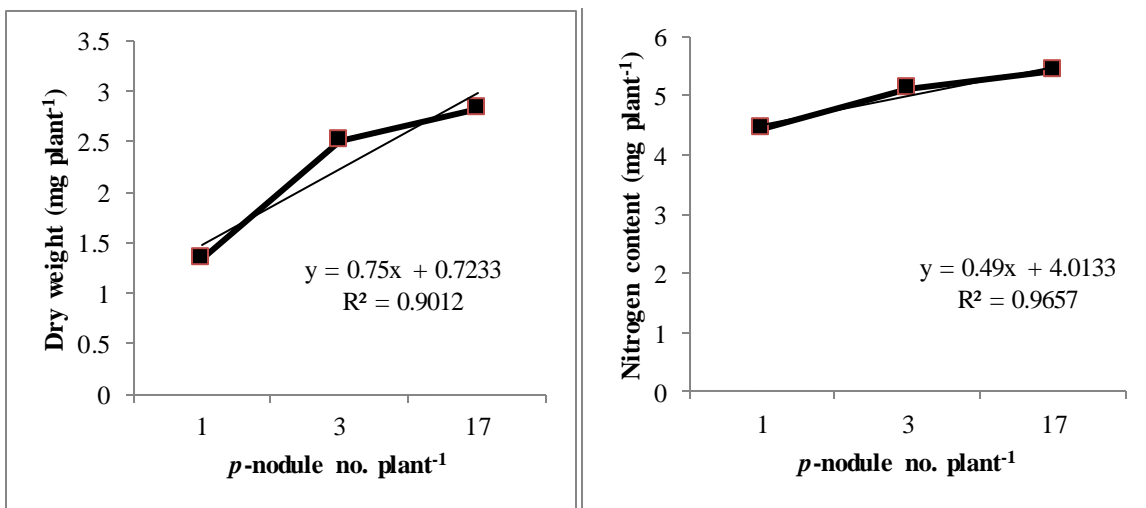
Fig. 4. Linear regression between p-nodule numbers and plant dry weight (A) and nitrogen content (B).

Fig. 5. Percentage increases in total biological (A) and protein (B) yields of cvs. Sakha 94 (I) and Giza 168 (II) over untreated plants. a) 100 % N, b) 50 % N, c) 50 % N plus Azospirillum and d) 50 % N plus Azospirillum plus IAA and enzyme mixture.

Table.1: Paranodulation and growth traits of 45-day old wheat seedlings due to chemical, enzyme and inoculation treatments

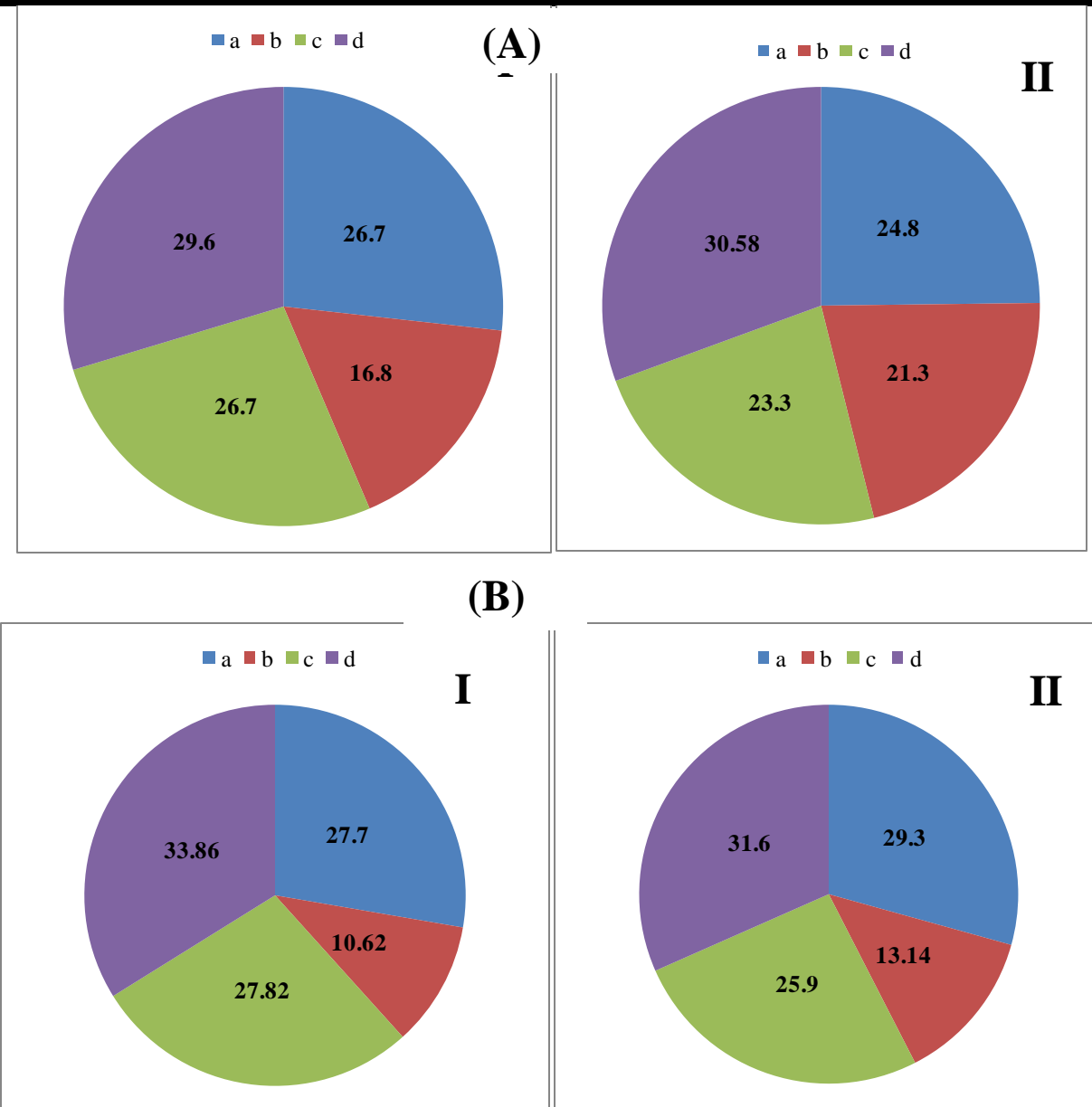| Treatments	p-nodule (no. plant^{-1})	Dry weight (mg plant^{-1})			N-content (mg plant^{-1})	
		Root (R)	Shoot (Sh)	R/Sh	Root	Shoot
cv. Sakha 94						
Control	0.0	4.6	14.9	0.31	0.08	0.31
2,4-D	18.0	4.4	12.8	0.34	0.06	0.26
IAA	12.0	6.1	19.4	0.31	0.09	0.38
Enz-PEG	8.0	3.9	13.5	0.29	0.06	0.24
Azospirillum(**Az**)	9.0	6.9	19.2	0.36	0.11	0.52
2,4-D + Az	22.0	7.2	20.4	0.35	0.08	0.46
IAA + Az	26.0	7.8	21.9	0.36	0.19	0.31
Enz-PEG + Az	19.0	6.8	20.1	0.34	0.09	1.00
cv. Giza168						
Control	2.0	3.9	11.3	0.35	0.06	0.19
2,4-D	14.0	3.7	11.9	0.31	0.07	0.10
IAA	19.0	5.5	14.2	0.39	0.08	0.21
Enz-PEG	12.0	3.2	10.9	0.29	0.08	0.19
Azospirillum(**Az**)	7.0	6.3	17.8	0.35	0.11	0.33
2,4-D + Az	19.0	6.4	17.6	0.36	0.10	0.39
IAA + Az	10.0	6.9	20.0	0.35	0.08	0.42
Enz-PEG + Az	23.0	4.8	12.8	0.38	0.06	0.24
cv. Gemeza10						
Control	0.0	4.1	12.0	0.34	0.04	0.12
2,4-D	9.0	4.9	10.6	0.46	0.03	0.09
IAA	6.0	5.2	13.9	0.37	0.05	0.16
Enz-PEG	12.0	3.3	9.9	0.33	0.03	0.13
Azospirillum(**Az**)	4.0	4.9	15.6	0.31	0.09	0.32
2,4-D + Az	18.0	4.8	11.1	0.43	0.08	0.25
IAA + Az	8.0	5.9	16.6	0.36	0.04	0.30
Enz-PEG + Az	16.0	4.9	10.4	0.47	0.05	0.19
LSD (0.05)	3.0	1.0	2.7		0.02	0.02
CV (%)	11.9	7.6	4.1		9.21	6.64

Table 2. Para-nodulation and growth parameters of 120-day old wheat plants treated with IAA and enzyme mixture in presence of N fertilizer and Azospirillum

Treatments	p-nodules (no. plant^{-1})	Dry weight (g plant^{-1})	N- content (mg plant^{-1})	Ear dry weight (mg plant^{-1})	Chlorophyll a (µg g^{-1}Dw)	Chlorophyll b (µg g^{-1}Dw)	Carotenoids (µg g^{-1}Dw)
Sakha 94							
Control	2.0	1.24	4.38	201.0	1.99	1.33	0.98
100 % N	2.0	2.89	5.93	690.0	2.13	1.88	1.71
50 % N+ IAA +Enzyme	22.0	1.26	4.26	401.0	1.88	1.73	1.66
50 % N + Azospirillum (Az)	16.0	2.57	5.18	582.0	1.91	2.02	1.32
50 % N+IAA +Enzyme +Az	34.0	2.99	5.87	692.0	2.08	2.45	1.44
Giza 168							
Control	0.0	1.41	4.51	344.0	1.86	1.62	1.01
100 % N	3.0	2.77	5.25	611.0	1.91	1.91	1.42
50 % N+ IAA +Enzyme	11.0	1.48	4.42	418.0	2.00	1.86	1.53
50 % N + Azospirillum (Az)	19.0	2.45	5.02	560.0	1.94	1.94	1.12
50 % N+IAA +Enzyme +Az	23.0	2.67	4.98	666.0	2.11	2.11	1.73
LSD (0.05)	4.0	1.13	0.20	167.0	0.19	0.19	0.14
CV (%)	6.6	4.61	9.52	14.9	8 .6	8.6	12.9

Table.3. Para-nodulation and yield parameters of field grown wheat cultivars of the different treatments

Treatments	p-nodules (no. plant^{-1})		TBY[a] (kg plot^{-1})		PY[b] (g plot^{-1})		GY[c] (g plot^{-1})	
	I	II	I	II	I	II	I	II
Without priming								
Control	4.0	6.0	0.96	0.84	19.8	16.8	386.1	323.2
100 % N	2.0	4.0	2.46	2.16	43.6	38.7	466.6	399.7
50 % N	6.0	8.0	1.93	1.88	28.4	26.7	401.4	321.6
50 % N + (Az)	11.0	7.0	2.29	2.02	44.2	37.9	515.5	476.3
50 % N+IAA +Enz. +Az	26.0	15.0	2.38	2.25	51.4	42.8	566.7	471.2
With priming								
Control	0.0	3.0	0.85	0.91	18.4	15.9	349.7	352.5
100 % N	0.0	12.0	2.51	1.99	46.7	48.2	488.8	412.1
50 % N	4.0	8.0	1.86	1.94	29.8	30.3	397.3	387.6
50 % N + (Az)	11.0	18.0	2.66	2.00	46.3	42.6	544.8	479.5
50 % N+IAA +Enz. +Az	19.0	33.3	2.92	2.47	50.6	48.4	571.4	441.7
LSD (0.05)	2.0		0.36		12.4		89.2	
CV (%)	8.4		18.9		16.5		11.3	

a, total biological yield; b, protein yield; c, grain yield.

Table.4: The calculated correlation matrix (R values) of the different parameters as affected by experimental treatments

	p-nodule no.	Plant Dw	Plant N-content	Ear dry weight	Chloro. a	Chloro. b	Carot.
p-nodule no.		+0.981^{ns}	+0.988*	+0.990^{ns}	+0.767^{ns}	+0.997*	+0.989^{ns}
Plant Dw			+0.991^{ns}	+0.998*	+0.628^{ns}	+0.993^{ns}	+0.894^{ns}
Plant N-content				+0.997*	+0.725^{ns}	+1.00**	+0.946^{ns}
Ear dry weight					+0.669^{ns}	+0.998*	+0.917^{ns}
Chloro. a						+0.716^{ns}	+0.910^{ns}
Chloro. b							+0.941^{ns}
Carot.							

Table.5. Change percentages in wheat grain yield (g plot^{-1}) due to seed water priming prior to cultivation (related to unprimed ones

Treatments	Sakha 94	Giza 168
Control	-9.4	+9.1
100 % N	+4.8	+3.1
50 % N	-1.0	+20.5
50 % N + *Azosp*	+5.7	+0.7
50 % N + *Azosp* + IAA-Enz	+0.8	-6.3