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Use of combined inoculum of *Azospirillum* and *Rhizobium* in winged bean *Psophocarpus tetragonolobus* (L) D.C.

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Summary

The potential of using the free living N -fixing Azospirillum in increasing the nodulation and N_2-fixation by Rhizobium *in winged bean was analysed. Various strains of winged bean* Rhizobium *and several strains of* Azospirillum *were tested in combined inoculations. Substantial increases in nodulation, N_2-fixation, shoot dry matter production and N gain due to the mixed inoculation were obtained in one* Rhizobium *strain namely KUL-BH and most of the* Azospirillum *strains used. The influence of Rhizobium genotype in expressing the association effect was more decisive than that of* Azospirillum. *The cell-free extract of* Azospirillum *produced the same enhancement effect as that of the organism itself, while the culture supernatant also showed the same in some instances. Since the response to combined inoculation in winged bean was obtained only with one strain of* Rhizobium, *the factors that could influence the nodulation enhancement were investigated in detail. An attempt was made in finding out the possible mechanism involved in the enhancement effect by* Azospirillum.

Samenvatting

De mogelijkheid werd onderzocht om vrij levende stikstofbindende Azospirillum*bakteriën te gebruiken om de wortelknolvorming en de stikstofbinding bij* Rhizobium *in gevleugelde boon (Psophocarpus tetragonolobus (L) D.C.) te bevorderen. Verschillende stammen van gevleugelde boon* Rhizobium *en verschillende stammen van* Azospirillum *werden getest in gecombineerde inoculaties. Een vermeerdering van nodulatie, stiksofbinding, droge stof der bovengrondse plantendelen en graanopbrengst werd bekomen met gemengde inoculatie waarbij Rhizobiumstam KUL-BH werd gebruikt in combinatie met de meeste* Azospirillum*stammen. Het effekt van* Rhizobium *genotype was belangrijker dan deze van* Azospirillum *in de uitdrukking van het associatief effekt. Gelvrij extrakt van* Azospirillum *vertoonde dezelfde voordelen als het organisme zelf. Aangezien het repons tot gecombineerde inoculatie bij gevleugelde boon werd verkregen met slechts een* Rhizobium*stam werden de factors onderzocht die de nodulatie beïnvloeden. Er werd een poging ondernomen om het mogelijk mechanisme te begrijpen dat het gunstig effekt van* Azospirillum *verklaart.*

Introduction

Studies on the role of the rhizobia in symbiotic specialization of winged bean *Psophocarpus tetragonolobus* (L) D.C. showed that there could be numerous effective, moderately effective and ineffective strains of winged bean *Rhizobium* present in different soil types of many winged bean growing countries (3). In the world-wide winged bean plant improvement programs, thousands of germplasm collections, new cultivars, bred varieties of varying origins and characters are being introduced into new growing areas. Such introduction of a new legume cultivar into soils containing a native population of rhizobia poses special demands for the rhizobial inoculant to be used. The performance of these introduced cultivars, varieties etc. will be highly dependent upon the compatibility of factors in host and rhizobia and the soil environment. Therefore use of the most effective strains of *Rhizobium* of winged bean is very important in attaining effective nodulation and N_2 fixation of this crop.

As a result of our screening studies on several strains of rhizobia which were isolated from different soils of many tropical countries, some effective wide spectrum winged bean *Rhizobium* strains are presently available. After attaining an effective symbiotic system with the use of such effective *Rhizobium* strains of winged bean, any further improvment of the symbiotic system requires new avenues. In the search of such new avenues, use of mixed inoculation of *Rhizobium* and some free living. N_2 fixing soil bacteria might be important. In this paper a review is made on the potential of using the free living N_2-fixing *Azospirillum* in increasing the nodulation and N_2-fixation of Rhizobium in winged bean.

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Out of free living, N_2 fixing soil bacteria, *Azospirillum* species is known for its root colonizing ability and its association with the root system of many plants including some crop cultivars. The free living associative symbiotic bacteria *Azospirillum brasilense* and *A. lipoferum* were found to cause substantial increases in total N content and yield of C-4 crop plants (6, 8, 11, 13). The inoculation of mixed cultures of *Azospirillum* and *Rhizobium* showed resultant increases in nodule number and grain yield of soybean, although these were not significant (10). There was significant enhancement effect on nodulation, N_2-fixation and N gain by several *Azospirillum* strains when combined pairwise with different *Rhizobium* strains in soybean (4). The similar free living associative symbiotic organism *Azotobacter vinelandii* caused enhanced nodulation when given as mixed inoculation with *Rhizobium* in soybean, cowpea and clover (1). Thus we undertook a study on the use of combined inoculation of *Rhizobium* and *Azospirillum* to incraese the nodulation and N_2 fixation of winged bean.

Material and methods

The strains of *Rhizobium* and *Azospirillum* used in the study were isolated from soil or plantroots collected from Belgium, Indonesia, Nigeria, Sri Lanka, Malaysia, Zaire and India (Table I).

TABLE I

Source of bacterial strains used in combined inoculation

Species and strain	Material of isolation	Country of soil
Rhizobium sp.		
Strain RRIM 56	Winged bean nodules	Malaysia
Strain NGR 258	Winged bean nodules	Malaysia
Rhizobium sp.		
Strain KUL-BH	Winged bean nodules	Indonesia
Strain KUL-JN	Winged bean nodules	Indonesia
Strain KUL-GP	Winged bean nodules	Sri Lanka
Strain KUL-Z3	Winged bean nodules	Zaire
Azospirillum brasilense		
Strain S63-1	Maize roots	Belgium
Strain KUL-X	Clayey soil	Nigeria
Strain KUL-Z	Roots of Musa sp.	Indonesia
Strain LUDHI	Loamy soil	India
Azospirillum lipoferum		
Strain KUL-Y	Winged bean root	Indonesia

Mixed cultures of combined inoculation were prepared by mixing equal volumes of *Rhizobium* and *Azospirillum* cultures of known densities (for details see Iruthayathas) (3).

Results and discussion

Substantial increases in nodulation, N_2 fixation, shoot dry matter production and N gain due to the mixed inoculation were obtained in combinations of one *Rhizobium* strain namely KUL-BH and most of the *Azospirillum* strains used (Table II). The influence of *Rhizobium* genotype in expressing the associative effect was more decisive than that of *Azospirillum*. The cell-free extract of *Azospirillum* produced the same enhancement effect as that of the organism itself, while the culture supernatant also showed the same in some instances (3). There was no enhancement effect by any of the *Azospirillum* strains on the *Rhizobium* strains RRIM 56 and NGR 258 (3, 4).

The *Rhizobium* strains KUL-GP, KUL-JN and KUL-Z_3 did not show any response to the combined inoculation, when one *Azospirillum* strain namely KUL-Y was tested (2, 3). However, we did not test the combinations of other *Azospirillum* strains and these three *Rhizobium* strains. Many strains of *Rhizobium* showed enhancement effect when combined with compatible *Azospirillum* strains in soybean (*Glycine max*) (3). However, in the case of winged bean, only one *Rhizobium* strain has shown the response to the *Azospirillum* combined inoculation. Therefore, the organisms seem to be showing certain specificity to produce the enhancement effect in the symbiosis. Among the *Azospirillum* strains, such specificity to the same *Rhizobium* strain was also shown. This was indicated by the varying symbiotic effectiveness of combined inoculation of KUL-BH with the different strains of *Azospirillum* (Table II). Strain KUL-Y, which showed the highest associative effect with *Rhizobium* in winged bean, was originally isolated from winged bean root.

Since the response to combined inoculation in winged bean was obtained only with one strain of *Rhizobium*, the factors that could influence the nodule enhancement were investigated in detail.

TABLE II

Nodule fresh weight (g), C_2H_2 reduction (nmol plant^{-1}h^{-1}), shoot dry weight (g), total shoot N content (mg) and symbiotic effectiveness after six weeks in winged bean selection $LBNC_1$ (For details of the experimental conditions see Iruthayathas et al., 1983 and Iruthayathas, 1984) with *Rhizobium* and *Azospirillum* combined inoculation and control.

Treatment	Nodule fresh weight*	C_2H_2 reduction*	Shoot dry weight*	Total shoot N content*	Symbiotic effectiveness+
RRIM 56 alone	10 79	34372	18 55	609 4	143
RRIM 56 + LUDHI	9.86	39561	16.28	590.8	125
RRIM 56 + KUL-Z	10 18	57964	14.88	476 4	115
RRIM 56 + KUL-Y	10.68	32378	17.65	667.0	136
RIM 56 + KUL-X	10.36	88390	15.93	549 0	123
RRIM 56 + CFE of KUL-X	4.98	15935	8.55	279.8	66
KUL-BH alone	5.76	31332	14.75	516.4	113
KUL-BH + LUDHI	7 26	47703	19.50	685 7	150
KUL-BH + KUL-Z	8.31	29865	20.15	711 6	155
KUL-BH + KUL-Y	8.64	82468	22.20	714 3	171
KUL-BH + KUL-X	8.73	66188	19 45	660.6	150
KUL-BH + CFE of KUL-X	8.79	52476	19.20	604 5	148
LSD (P = 0 05)	2.30	22983	5.01	168.3	

* significant at 0.1%.
+ Shoot dry weight of treatment expressed as a percentage of N control plants (see Iruthayathas, 1982)

First of all, the effect of methods of combined inoculation on the success of enhancement effect was studied. Usually the inoculation of a mixture of *Rhizobium* and *Azospirillum* cultures was done at planting. The seeds were pregerminated and uniformly germinated seedlings were selected and surface inoculated with 1 week old turbid suspension of a mixture of broth cultures of *Rhizobium* and *Azospirillum*. Also 2 days after emergence of seedlings the same inoculum was applied to the soil. We tested the effect of form of bacterial cutlures on the nodulation enhancement (3). Regardless of the form, whether peat culture or broth culture the enhancement effect was seen in combined inoculation. Time of application of inoculum was also checked for its effect on the nodulation enhancement. Seed inoculation with *Rhizobium* at planting was done and the *Azospirillum* inoculation was given 1 week after emergence of seedlings. In such delayed inoculation of *Azospirillum* also the nodulation enhancement was seen.

Since we have seen that the methods of combined inoculation had little effect on the success of nodulation enhancement effect we investigated the effect of cell free extract (CFE) of *Azospirillum* on the symbiosis of winged bean.

This study was specially important, because the CFE of *Azospirillum* strain KUL-X drastically reduced nodulation and N_2 fixation of *Rhizobium* strain RRIM 56 (Table II). In repeated experiments we found that none of the *Azospirillum* strains in the form of viable cells had any effect on this *Rhizobium* strain (Iruthayathas, 1984), but the cell free extracts of all the *Azospirillum* strains reduced the nodulation and N_2 fixation (Table III). It was also found that this reduction effect of CFE in this particular *Rhizobium* strain was through the reduction of root mass of the plant. The same effect was shown by free glutamic acid while aspartic acid did not. Thus glutamic acid may be the cause alone or along with other components of the CFE of *Azospirillum*. The first amino acid formed during the dinitrogen fixation of *Rhizobium* is glutamic acid. Thus, if glutamic acid is readily available to the host plant from the CFE inoculation, during the *Rhizobium* infection and nodule initiation period, the host plant may not favour the nodule formation processes.

However, it is still puzzling that such inhibition/reduction of nodulation by the CFE of *Azospirillum* was not encountered in other *Rhizobium* strains of winged bean.

The possible mechanism involved in the nodulation enhancement effect by *Azospirillum* was investigated to a certain extent. In a similar combined inoculation study but with *Azotobacter* in cowpea, clover and soybeans, Burns et al. (1) suggested that enhanced nodulation by *Azotobacter* was mainly through it influencing nodule initiation and not nodule growth or function.

TABLE III

Nodule fresh weight (g), C_2H_2 reduction (nmol plant^{-1}h^{-1}), shoot dry weight (g), root dry weight (g) and symbiotic effectiveness (%) after sixth week in winged bean *Rhizobium* strain RRIM 56 and CFE of *Azospirillum* inoculation.

Treatment	Nodule fresh weight*	C_2H_2 reduction*	Shoot dry weight*	Root dry weight*°	Symbiotic effectiveness (%)+
RRIM 56 alone	6.26a	7424a	7.94a	2.60a	73
RRIM 56 + CFE of KUL-Y	0.33b	75b	2.71b	1.46b	25
RRIM 56 + CFE of KUL-Z	0.08b	208b	2.56b	1.42b	24
RRIM 56 + CFE of KUL-X	2.23b	2181b	3.96b	1.92b	37
RRIM 56 + CFE of LUDHI	8.70a	8619a	10.48a	2.96a	97
RRIM 56 + Glutamic acid	0.86b	1161b	2.59b	1.61b	24
RRIM 56 + Aspartic acid	8.10a	8000a	9.42a	2.69a	87
LSD (P = 0.05)	3.13	2778	3.07	0.68	

* Significant at 0.1%. Values followed by common letters are not significantly different from one another.

° Coefficient of correlation (r) between root dry weight and nodule weight was 0.92 (significant at 0.1 %).

+ % of uninoculated plant with weekly application of 46 mg N.

However, in most of our experiments there was no response in nodule number due to mixed inoculation but these experiments showed enhanced nodule mass and N_2 fixation due to mixed inoculation. Since the response to mixed inoculation was shown in nodule mass, N_2 fixation, N gain and plant dry matter, *Azospirillum* would appear to influence the development and function of the nodule after initiation. This is further supported by the fact that only in the nodules of effective combined inoculation treatments *Azospirillum* bacteria were present in large numbers (when isolated from surface sterilized crushed nodules). Also in the non-effective combination of RRIM 56 with various *Azospirillum* strains, *Azospirillum* althrough present in the roots, was not present in the nodules.

Burns et al. (1) reported that the possible agent responsible for the enhancement effect by *Azotobacter*, may be a protein or an enzymatic product present in the cells of *Azotobacter*, and that compound may not be excretable. Their suggestion is in agreement to a certain extent with our results, as the cell free extracts of *Azospirillum* produced the same effect as the organism itself. However, it should be noted that the culture supernatant also produced a similar effect in many of our experiments. The culture supernatant contains some plant growth substances, tryptophan and tryptamine (Table IV). An important characteristic of *Azospirillum* is its ability to convert tryptophan into indole acetic acid (9), a substance that promotes the infection process of *Rhizobium* in root hair (7).

TABLE IV

Amounts (nmol 1⁻¹) of coumpounds present in culture supernatant of *Azospirillum* S.63-1, 1-7 days after inoculating the medium with bacteria

Days after inoculation	Tryptophan	Tryptamine	Indole Pyruvic acid	Indole acetic acid	Indole propionicd acid	Indole carboxylic acid
1	41.56	276.1	68.00	65.06	78.56	38.57
2	12.12	113.93	28.80	11.63	89.67	59.85
4	32.47	120.22	60.00	16.39	39.28	46.55
5	22.08	130.01	66.40	34.38	58.92	19.95
6	58.88	118.83	79.20	0.00	170.80	90.44
7	29.87	88.07	40.00	0.00	64.90	30.59

Iruthayathas et al. (5) indicated that for nodule formation, *Rhizobium* strain NGR 258 was sensitive to light intensity and this sensitivity may be related to the tryptophan availability in the root hair for the conversion into IAA.

Wathever the agent(s) responsible for the enhancement effect, it must be influencing the root hair formation, as indicated by the remarkable variation in nodule distribution. Tien et al. (12) found many indole compounds (including IAA), gibberellin like substances, 3 cytokinin like substances in the culture supernatant of *Azospirillum*. They also showed that the morphology of pearl millet roots changed when plants were inoculated with *Azospirillum*. The number of lateral roots was increased and all the lateral roots were densely covered with root hairs. When they tested with pure plant hormones, combinations of IAA, GA_3 and kinetin (at very low concentration) produced the same changes in root morphology as that by *Azospirillum* inoculation. When we tested the application of pure IAA, adenine and kinetin in combination with KUL-BH *Rhizobium* strain no significant response was noticed. But the concentrations and the combinations of these pure hormonal compounds in our experiments may not be the appropriate levels to get a response. In a colony plate count experiment, we found that the hormonal compounds in our experiments when present in supernatant part of *Azospirillum*, remarkably increased the rhizobia population (Table V). We should also emphasize that CFE also showed such increase in rhizobia population and this effect may probably be due to substances such as adenine (6-amino purine) which shows an action similar to cytokinin. Antoher important point is that kinetin (6-furfuryl purine) is usually prepared from degraded DNA adenine and it may be possible that while sonicating the *Azospirillum* cells to obtain cell free extracts, the degradation of DNA adenine into kinetin may take place. In other words, in CFE too, the hormonal compounds could be present.

Thus the only inference we could make from these studies would be that the hormonal compounds produced by *Azospirillum* organism or from its CFE, might have increased the infection sites by increasing the number of root hairs in the plant and the number of rhizobia in the rhizosphere.

TABLE V

Number of rhizobia⁺ (in 10^9) when grown in mixed cultures with *Azosprillum* strain KUL-X and its CFE and supernatant.

Treatment	Number of rhizobia of RRIM 56 ***	number of rhizobia of KUL-BH ***
Rhizobium alone	101a	250a
Rhizobium + *Azospirillum* strain KUL-X	111a	259a
Rhizobium + CFE of *Azospirillum* strain KUL-X	196b	335b
Rhizobium +Supernatant of *Azospirillum* strain KUL-X	194b	336b
LSD (P = 0.05)	37	38
Number of *Azospirillum* in *Rhizobium* + *Azospirillum* treatment	122	150

+ Each value was the mean of 24 plate counts.
*** Significant at 0.1 %.

Yet, we still have to reason out as to why an enhancement response was not obtained with *Rhizobium* strain RRIM 56 which also showed increase in rhizobial number when *Azospirillum* CFE and culture supernatant were given. It may be possible that once the infection sites are increased by *Azospirillum* and minute nodules are formed in increased number, the enlargement of nodule takes place at an increased pace by the effect of some non-excretable compounds (compounds other than hormonal and similar substances in this context) of *Azospirillum* in the case of KUL-BH combined inoculation. In the case of RRIM 56 combined inoculation, these compounds did not cause any enhancement due to unknown complex reason.

Conclusion

The critical evaluation of the use of *Azospirillum* and *Rhizobium* combined inoculation in winged bean, based on the current data, indicates that this practice may be adopted only for a limited number of *Rhizobium* strains. However, the indirect effects of combinations of these two species of bacteria, such as a possible increase in the number of flowers and therby an increase in grain yield have not been tested in winged bean. Another such possible indirect effect would be an advance in the time of flowering. These studies require field experimentation.

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