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Intrinsic Antibiotic Resistance, Survival of *Rhizobium Leguminosarum* Strains and Fixation Potential of Pea Varieties (*Pisum sativum* L.) In Southeast Ethiopia

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Abstract: A study to examine the effects of genotype x *Rhizobium leguminosarum strain* for their antibiotic resistance and survival of the introduced *rhizobium* strains and nitrogen fixation potential on pea varieties was conducted in soils of Sinana in 2005. Two strains (EAL 300 and EAL 302) of *Rhizobium leguminosarum* and their combination were evaluated in three pea varieties. Nodule occupancy of inoculated *rhizobia* was determined by their sensitivity to different levels of concentrations of antibiotics in pure culture and then comparison of antibiograms of isolates from nodules. It was observed that strain EAL 302 was highly competitive occupying 40, 50 and 30% nodules in local, 'Wayitu' and 'Dadimos' varieties of field pea. Similarly, study of survival of *rhizobia* on seeds indicated maximum number of *rhizobial* cells due to strain EAL 302 on inoculated seeds of variety 'Wayitu' and local. However, variety 'Dadimos' was supported the least number of *rhizobia* per seed. Based on the findings it could be suggested that inocula of strain EAL 302 on field pea variety 'Wayitu' was the best combination with respect to response in terms of resistance to antibiotic and survival rate of strain on seeds which there by enhance nitrogen fixation.

Key words: Concentration · Competitive · Ethiopia Agricultural Legumes (EAL) · Genotype

INTRODUCTION

The challenge facing research on increasing food production in tropical Africa now is to develop low input systems that are not only sustainable but also economically viable. The main value of legumes, in this aspect in building up soil organic nitrogen to the level that adequate amount can be made available for future crops, is remarkable [1]. Thus, inoculation with an effective and persistent *rhizobium* strain has numerous advantages, which includes non-repeated application of nitrogen fertilizers [2].

Survival of applied *rhizobia* on seed and nodulation in the field is generally more effective when solid based inoculants are applied onto the seed than with other forms of inoculants. When regulations exist, minimum standards at the time of inoculant expiry are 10^6 to 10^8 cells/g of inoculant. The most widely accepted standard for numbers of *rhizobia* per seed are 1000. Burton [3] has recommended 1000 for fast-growing *rhizobia* and 100,000 for slow-growing *rhizobia*. The safest quality standards could be applied to inoculated seed, where 10^5 rhizobia per large seed (e.g., soybean, lupin), 10^4 *rhizobia* per medium seed (e.g., mungbean, pigeonpea) and 10^3 per small seed (e.g., clover, siratro) are required.

In order for proper nodulation to occur, effective inoculation needs to happen to maintain high numbers of viable bacteria until such time as they can nodulate the roots. However, the formation of an effective symbiosis requires the presence of strains of *rhizobium* in the soil that can nodulate the host legume. Contrary to this, effective *rhizobial* strains are often either not present in soils or are present in only very low numbers and must be made available to farmers as seed inoculant [4]. To date, very limited information has been available on BNF research in Ethiopia [5].

In the present study, therefore, an attempt has been made to assess the antibiotic resistance and survival of the inoculated strain on seeds. In view of the above facts, the present study was carried out with objectives to notify the inherent resistance of the introduced strains to the different standardized antibiotic concentrations for their effectiveness in relation to nodulation pattern and N_2-fixation and to determine the survival rate of *rhizobium* population on inoculated seeds of field pea.

MATERIALS AND METHODS

The experiment was conducted on silty-loam soil in green house under continuous cereal dominated

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production systems in 2005 cropping season and further study was made in the microbial lab center. The sample soil taken is situated within 07° 07' N latitude and 40° 10' E longitude at an altitude of 2400 m a.s.l. The area is typically characterized by bimodal rainfall having two distinct growing season *viz., 'Ganna'* (March-July) and *'Bona'* (August-December).

Carrier peat based inoculants of *Rhizobium leguminosarum* strains EAL 300 and EAL 302, to study their effect on pea varieties, were obtained from Soil Microbiology Laboratory of National Soil Research Center (NSRC), Addis Ababa, Ethiopia. Pure cultures of same strains were also collected for determination of antibiotic resistance of the strains. During the course of study, experiments on intrinsic antibiotic resistance of *rhizobial* strains, survival of *rhizobia* on inoculated seeds of different pea varieties were conducted.

Survival of *Rhizobia* on Seeds: Two strains of *Rhizobium leguminosarum* and their combination namely EAL 300, EAL 302 and EAL 300+ EAL 302 and three varieties of field pea namely local, 'Wayitu' and 'Dadimos' were tested for survival of *rhizobia* on seeds. Seeds of the three varieties of field pea were inoculated with equal amount (7 g/kg seed) of inoculant of different strains of *Rhizobium leguminosarum* and their combination. The inoculated seeds were incubated at 30 °C for 10 days. Population of *Rhizobium leguminosarum* strains was determined by making serial dilutions of the inoculated seeds and then plating 1ml of diluted sample on YEMA medium (yeast extract manitol agar) at 0, 1, 2, 3, 4, 5, 6, 8 and 10 days intervals. The number of *rhizobia* on seeds were reported as cfu/seed.

Intrinsic Antibiotic Resistance (IAR) Pattern of *Rhizobial* Species: Intrinsic antibiotic resistance (IAR) study was undertaken in the laboratory by preparing stock solutions (10 mg/ml) of the antibiotics in the appropriate solvent (Table 1), filter sterilized and stored at 4°C. YEMA medium was prepared by dispensing 50ml in 100ml conical flasks and autoclaved at 15 lbs psi for 30 minutes. The medium was cooled to 50-60°C and appropriate concentration of antibiotic was added.

Table 1: Antibiotics and their solvents

Antibiotics	Solvents
Ampicillin	Distilled water
Vancomycin	Distilled water
Tetracycline	50% ethanol

Ampicillin and Vancomycin were added at concentrations 100, 125, 150 and 200 μg ml^{-1} whereas tetracycline was tested at 5, 10, 15 and 25 μg ml^{-1}. The melted medium with the respective concentrations of different antibiotics was pored into two plates for each concentration and allowed to solidify. Pure cultures of each strain were streaked on the antibiotic plates and control plates (no antibiotic). The inoculated plates were incubated for 48-72 hours at 30°C. After incubation, the growth on antibiotic plates was compared with control plates. Growth was registered as positive (+++, ++, +) or negative (-) for each concentration. The resistance limit was considered up to that concentration beyond which growth did not occur.

The intrinsic antibiotic resistance of the *rhizobia* isolated from root nodules of plants from treatments in which both strains had been used, was done by selecting 10 nodules at random from each treatment and making isolations from each. The pure cultures of each isolate were tested for antibiotic resistance as described above. The percentage nodule occupancy was determined by comparing the antibiogram (concentration of each antibiotic at which growth of a *rhizobial* strain/isolate did not occur) of pure strain with that of isolates from nodules from each treatment. The number of isolates from nodules of each treatment matching the antiobiogram of any of the two strains were taken as the number of nodules out of total 10 nodules occupied by that strain. Accordingly, the percentage nodule occupancy by each strain was calculated.

RESULTS

Survival of *Rhizobial* Strains on Seeds: The present study involved determination of viable living cells on inoculated seeds at various days of interval. The results of experiment are reported in Figure 1, 2 and 3. The survival of *rhizobia* on seeds of field pea varieties indicated variations in terms of *rhizobial* number among the inoculated seeds at different time intervals. Maximum number of cells were exhibited on the seeds of variety 'Wayitu' and local variety on the first day of inoculation. Relatively variety 'Dadimos' was found less effective in maintaining more number of viable cells on seed. Strain EAL 302 inoculated on pea varieties, 'Wayitu' and local, recorded the maximum number of *rhizobial* cells ($7x10^4$ cfu/seed) immediately after inoculation. On the other hand, combination of strain (EAL 300+302) showed maximum number of cell colony ($6x10^4$ cfu/seed) on seeds of variety 'Wayitu'. The number of *rhizobia* on seeds started declining after first day of inoculation.

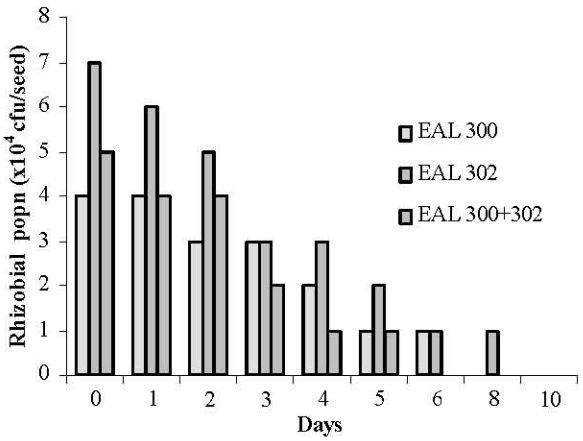

Fig. 1: Survival of *rhizobia* on seeds of local variety

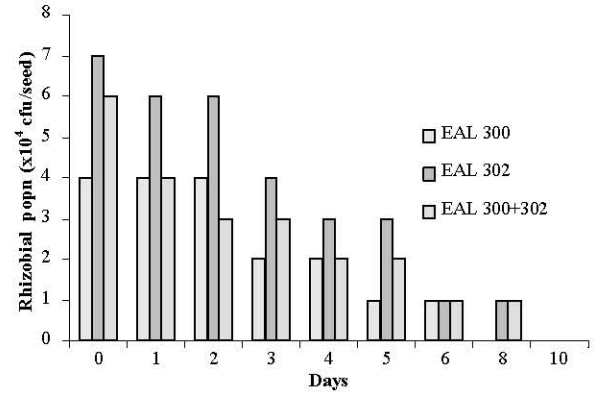

Fig. 2: Survival of *rhizobia* on seeds of 'Wayitu' variety

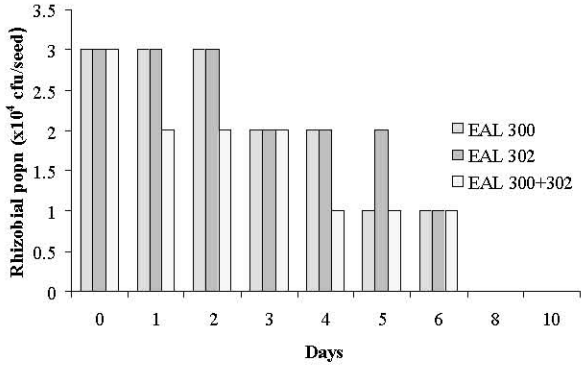

Fig. 3: Survival of *rhizobia* on seeds of 'Dadimos' variety

However, it was interesting to note that enough population of strain EAL 302 was maintained on seeds of varieties 'Wayitu' and local at the end of 8 days, whereas *rhizobia* on seeds of 'Dadimos' could not be detected after 6th day of incubation. The above findings were in the safest quality standards required for inoculated seeds immediately after inoculation. Thus, all inocula resulted in the safest quality standards required for inoculated seeds. However, the presence of enough number of viable cells for a longer period after inoculation on the seeds of variety like 'Wayitu' will ensure better establishment of *rhizobia* once the seeds are sown.

Therefore, less number of *rhizobial* cells observed in case of EAL 300 in comparison to EAL 302 and strain combination (EAL 300+302) indicated that EAL 300 survived to lesser extent on seeds after inoculation which may indirectly affect its performance under field conditions.

Intrinsic Antibiotic Resistance of *Rhizobial* Strains: Pure culture strains of EAL 300 and EAL 302 were tested for their resistance to different antibiotics at various levels of concentration. As shown in Table 2 strain EAL 300 was sensitive, in other words did not show any growth, at 5 μg ml^{-1} of tetracycline, 125 μg ml^{-1} of ampicillin and 125 μg ml^{-1} of vancomycin. But, EAL 302 was sensitive to 25 μg ml^{-1} of tetracycline, 125 μg ml^{-1} of ampicillin and 125 μg ml^{-1} of vancomycin. Accordingly following antibiograms of two strains were identified:

EAL 300 _ $Tetracyclin_{5}Ampicillin_{125}Vancomycin_{125}$
EAL 302 _ $Tetracyclin_{25}Ampicillin_{125}Vancomycin_{125}$

Percent nodule occupancy of each strain was then determined by isolating *rhizobia* from 10 randomly picked nodules from treatments in which combined inoculation of EAL 300 and EAL 302 had been done (Table 3). The isolates from each treatment were also examined for their antibiotic resistance in similar fashion as described above and their antibiograms were matched with that of pure cultures of EAL 300 and EAL 302.

Table 2: Sensitivity of *rhizobial* pure culture strains to different concentrations of antibiotics

	Antibiotics (concentration in μg ml^{-1})											
	Tetracycline				Ampicillin				Vancomycin			
Strains	5	10	15	25	100	125	150	200	100	125	150	200
EAL 300	-	-	-	-	+	-	-	-	+	-	-	-
EAL 302	+++	+++	++	-	+	-	-	-	+	-	-	-

+++ Good growth, ++ Moderate growth, + Fair growth, - No growth

Table 3: Nodule occupancy as affected by the different strains

	Strains		
Variety	EAL 300	EAL 302	Other
Local	20%	40%	40%
Wayitu	15%	50%	35%
Dadimos	25%	30%	45%

On the basis of number of nodules occupied by each strain, it was observed that strain EAL 302 was highly competitive occupying 40, 50 and 30% nodules in local, 'Wayitu' and 'Dadimos' varieties of field pea, respectively. Compared to other varieties, 'Wayitu' seemed to be more responsive to infection by inoculated strain EAL 302. However local variety and 'Dadimos' seemed to be more adapted to the infection by native *rhizobia* in the soil. Generally, it can be deduced strain EAL 300, in terms of nodules occupied in the roots of three varieties of field pea, was less competitive under field conditions than EAL 302 and native *rhizobia*.

DISCUSSION

The survival of inoculated *rhizobia* on seed may be considered an important criterion for selecting effective commercial strain(s). Therall *et al.* [6] briefly discussed in his recent research work that *rhizobial* inoculation by seed coating technique increased the establishment rate more than double and often significantly increases seedling growth rates. Survival of *rhizobia* is thus an important criteria limiting nodulation of legumes. Different varieties have different rate of survival of *rhizobial* strains on their seeds. In the present study considering various pea varieties, strain EAL 302 inoculated on pea varieties like 'Wayitu' and local recorded maximum number of *rhizobial* cells (7×10^4 cfu/seed) immediately after inoculation. However, the persistent of *rhizobial* cells were still maximum in case of EAL 302 in 'Wayitu' variety as compared to other so important. The above findings were in agreement with Burton [3] who stated the safest quality standards required for inoculated seeds were 10^5 *rhizobia* per large seed (soybean, lupin), 10^4 *rhizobia* per medium seed (mungbean, pigeonpea) and 10^3 per small seed (clover, sirato) immediately after inoculation. In studies of *rhizobial* survival following aerial seeding, Hely [7] demonstrated that inoculation and seed coating produced vigorous swards of well-nodulated, N_2-fixing crimson clover (*Trifolium incarnatum*). Similarly, recent research work done by Thrall *et al.* [6] has shown that *rhizobial* inoculation (by seed coating) of direct-seeded acacias more than doubles their establishment rate and often significantly increases seedling growth rates. The less number of *rhizobial* cells observed in case of EAL 300 in comparison to EAL 302 and strain combination (EAL 300+302) indicated that EAL 300 survived to lesser extent on seeds after inoculation which may indirectly affect its performance under field conditions. Evans *et al.* [8] also reported that poor survival of symbiotic N_2 fixing soil bacteria might lead to unsatisfactory nodulation of legumes, reducing their productivity.

In practice the efficacy of an inoculant depends on its efficiency for N_2-fixation but also on other aspects of effective inoculation technology that are influenced by strain. Thus, survival of inoculant on seed is one of the most important of these, because poor survival may lead to reduced nodulation that in turn may reduce pulse yield Roughley *et al.* [9]. It is for the fact that persistence of strains from season to season is generally a desirable trait in annual legumes as it makes yearly inoculation unnecessary. Pure culture strains of EAL 300 and EAL 302 tested for their resistance to different antibiotics at various levels of concentrations showed EAL 302 was relatively more resistance as compared to EAL 300 and their combination. On the basis of which strain EAL 302 was highly competitive occupying 40, 50 and 30% nodules in local, 'Wayitu' and 'Dadimos' varieties of field pea. Such findings corroborated by Materon *et al.* [10] and he stated as symbiotic nitrogen fixation rates could be markedly increased by highly efficient, competitive and persistent strains of *rhizobia*.

Compared to other varieties, 'Wayitu' seemed to be more responsive to infection by inoculated strain EAL 302 while strain EAL 300, in terms of nodules occupied in the roots of three varieties of field pea, was less competitive under field conditions than EAL 302 and native *rhizobia*. So, varietal response is expected in terms of N_2-fixation efficiency reported by several researchers. For instance, a linear and positive effect of increased N_2 fixation on seed yield was observed in inoculation assays with *rhizobial* strains of various N_2 fixation efficiencies Ravuri and Hume [11]. Early investigation of the effect of RT strains, major component of *Bradyrhizobium* populations on the host soybean plant revealed that genotypic variability exists in the response to nodulation Erdman *et al.* [12].

In conclusion, from the findings it can be deduced significance fixation potential is clearly observed owing in variety 'Wayitu' combined with strain EAL 302. It is therefore varietal selection to the best matching microbial organisms is paramount important for sustainable agricultural production and resource management.

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REFERENCES

1. Mulongoy, K. and M.K. Van der Meersch, 1988. Nitrogen contribution by leucaena (*Leucaena leucocephala*) prunings to maize in an alley cropping system. Biological Soil Fertility, 6: 282-285.
2. Sanginga, N., S.K.A. Danso, K. Mulongoy and A.A. Ojeifo, 1994. Persistence and recovery of introduced *Rhizobium* ten years after inoculation on *Leucaena leucocephala* grown on an Alfisol in Southwestern Nigeria. Plant and Soil., 159: 199-204.
3. Burton, J.C., 1979. New developments in inoculating legumes. pp. 380-405. *In:* N.S. Subba Rao (Ed.) Recent Advances in Biological Nitrogen Fixation. Oxford Press, New Delhi.
4. Marufu, L., N. Karanja and M. Ryder, 1995. Legume inoculant production and use in east and southern Africa. Soil. Biology and Biochems., 27(4/5): 735-738.
5. Desta Beyene, 1988. Biological Nitrogen Fixation Research on Grain Legumes in Ethiopia-An Overview. *In:* (Beck, D.P. and L.A. Materon, eds.). Nitrogen Fixation by Legumes in Mediterranean Agriculture: Proceedings of the workshop on Biological Nitrogen fixation by Legumes on Mediterranean-type Agriculture 14-17 ICARDA, Syria.
6. Thrall, P.H., D. Millsom, A.C. Jeavons, M. Waayers, G.R. Harvey, D.J. Bagnall and J. Brockwell, 2005. Studies on land restoration: seed inoculation with effective root-nodule bacteria enhances the establishment, survival and growth of *Acacia* species. J. Applied Ecol., pp: 42.
7. Hely, F.W., 1965. Survival studies with Rhizobium trifolii on seed of Trifolium incarnatum L. inoculated for aerial sowing. Australian J. Agricultural Res., 16: 575-589.
8. Evans, J., C. Wallace and N. Dobrowolski, 1993. Interaction of soil type and temperature on the survival of Rhizobium leguminosarum bv. viciae. Soil Biology and Biochemis., 25(9): 1153-1160.
9. Roughly, R.J., L.G. Gemell, J.A. Thompson and J. Brockwell, 1993. The number of *Bradyrhizobium* sp. (*Lupinus*) applied to seed and its effect on rhizosphere colonization, nodulation and yield of lupin. Soil Biology and Biochemistry, 25: 1453-1458.
10. Materon, L.A., J.D.H. Keatinge, D.P. Beck, N. Yurtsever and S. Altuntas, 1995. The role rhizobial biodiversity in legume crop productivity in the west Asian highlands II. Rhizobium leguminosarum. Experimental Agric., 31: 485-491.
11. Ravuri, V. and D.J. Hume, 1993. Soybean stover nitrogen affected by dinitrogen fixation and cultivar. Agronomy J., 85: 328-333.
12. Erdman, L.W., H.W. Johnson and F. Clark, 1957. Varietal responses of soybeans to a bacterial-induced chlorosis. Agronomy J., 49: 267-271.