Report of the scientific activity of Dr Andrey Belimov
during the period spent at the Sassari Section of ISPAAM-CNR
in the frame of the 2009 CNR- Short Term Mobility Program

1. Introduction

Collaboration between ARRIAM and ISPAAM-CNR has been established ten years ago. During that period two joint research projects within the Italy-Russia Commission for Science and Technology Cooperation have been completed. The titles of these projects were: "Isolation, characterization and utilization of root nodule bacteria specific to pasture legumes belonging to the genera *Anthyllis, Astragalus, Biserrula, Lotus, Hedysarum, Hippocrepis, Ornithopus, Scorpiurus* and selection of high effective strains" and "Development of symbiotic systems between pasture legumes and soil microbes for restoring fertility of polluted and arid soils". The results obtained during collaboration has been presented at different International conferences and published in refereed journals, such as Antonie Van Leeuwenhoek (Safronova et al., 2004), Soil Biology and Biochememistry (Belimov et al., 2005) and FEMS Microbiology Letters (Safronova et al., 2007). At the same time a big portion of the results has not been published, because some aspects needed to be discussed in more detail, some experiments and analyses needed to be completed and finalised, and possibilities for development of collaborative research must be evaluated. Therefore the CNR-Short Term Mobility Program gave an unique possibility for significant progress in collaboration and benefited both partners.

2. Activities done in the period from 08.06.2009 to 20.06.2009:

2.1. Round-table discussions

Two round-table discussions were organized with CNR researchers of ISPAAM, ISPA and ICB institutes about topical problems related to plant physiology, microbiology, agriculture and environment. At the first meeting, mechanisms of heavy metal tolerance in plants, stressing on the microbiologic aspects of plant-microbes-soil systems were discussed, and activities of ARRIAM aimed on this issue were introduced by Dr. Andrey Belimov. The second meeting was aimed at discussion about the role of plant growth-promoting rhizobacteria (PGPR) containing enzyme ACC deaminase in adaptation of agricultural crops

to environmental stresses, particularly drought stress. Dr. Andrey Belimov, being an expert in ACC deaminase containing rhizobacteria, described the mechanism of this plant-bacteria interaction, outlined current state of the art in the study of ACC deaminase, and presented his recent results (Belimov et al., 2009 and unpublished data) about interactions between plants and ACC deaminase containing bacteria under drought stress. Since this type of PGPR was one of the subjects of cooperation between ARRIAM and ISPAAM-CNR, such discussion was very important for participants in order to generalise the results obtained and to plan future experiments. The main conclusions from the meetings were: (I) It is a very promising approach to consider plant-microbe-soil continuum as a complex and fine functioning biological system having over-organism properties and tuning for maintaining homeostasis; (II) For improvement of plant adaptation to stressful environments it is necessary to use potential of beneficial plant-associated microorganisms.

2.2. Expedition

A local expedition was organised to collect samples from the roots of plants inhabiting coastal environments and subjected drought and saline stress (Fig. 1). The root samples of legume plants of *Lotus cytisoides* were collected from six places of the north Sardinia coast for further inspection of the presence and isolation of ACC deaminase containing rhizobacteria. *L. cytisoides* is a perennial prostrate species with yellow flowers, very widespread in coastal rocky areas also on gravels and sometimes on sand (Pignatti, 1982). Its prostrate growth, perenniality, resistance to drought stress and the adaptation to very poor soils contribute to justify the interest about the species for uses in stressful environments for soil protection on slopes of Mediterranean areas (Meloni et al., 2000). It is also an interesting species for studies about interactions between legume species and rhizosphere microrganisms on sandy and rocky coastal environments, useful to better exploit the potential of the species in recovery actions on coastal areas degraded by anthropic frequentation and activities.

2.3. Training course in microbiology

Training of ISPAAM researchers on isolation and purification of ACC deaminase containing bacteria from the rhizosphere and plant roots was carried out by Dr. Andrey Belimov. This kind of beneficial bacteria can utilize ACC, the immediate precursor in biosynthesis of phytohormone ethylene, as a nutrient source (Glick et al., 1997; Belimov et al., 2005).

Bacteria that have ACC deaminase are capable of stimulating plant growth and the mechanism of bacterial effect is as follows. Some of the plant ACC is exuded from roots or seeds, taken up by the bacteria, and then cleaved by ACC deaminase to ammonium and α-ketobutyrate (Fig. 2). The reduction of ACC levels within the plant results in a decrease in the ethylene content and leads to elimination of plant growth inhibition caused by this hormone (Glick et al. 1998; Belimov et al., 2001). Since biosynthesis of ethylene increases under various unfavourable environment conditions, including heavy metal toxicity, drought and salinity, the growth-promoting effect of ACC-utilizing rhizobacteria maybe of particular importance in the presence of stresses.

The collected root samples of the legume plant *Lotus cytisoides* were used for isolation of ACC-utilizing bacteria. Application of selective nutrient media containing ACC as a sole source of nitrogen (Belimov et al., 2001, 2005) showed that such bacteria are present in all root samples collected. Example of this procedure is given on Figure 3. As a result of this course the ISPAAM researchers have obtained new and necessary experience for selection of beneficial microorganisms having specific properties in plant growth promotion under stressful conditions. During the course more than fifteen bacterial strains and one fungal strain have been isolated. Dr Giannella Piluzza from ISPAAM is now continuing isolation and purification procedures. At the second step of this work determination of ACC deaminase activity of bacterial strains by biochemical assay and identification up to a species level by sequencing the 16S rRNA gene will be performed. The isolated bacteria will enlarge the bioresources for future research and will give valuable information about biodiversity of microorganisms present in the studied environments. The isolation technique can be easily adapted and used for the study of beneficial microorganisms inhabiting the rhizosphere of various plant species and soils.

2.4. Establishing of new collaborative research activity

During fruitful communications at the round-table discussions about tolerance of plants to heavy metals, new ideas for scientific cooperation were developed by Drs. Andrey Belimov, Simonetta Bullitta and Guy d'Hallewin. It has been agreed to start a new collaborative activity related to the study of transport and compartmentalization of toxic cadmium in plant tissues and plant cells. The main topic of the planned research is a cadmium tolerant and cadmium accumulating mutant of pea SGECdt (Fig. 4), which has been recently isolated by Dr. Andrey Belimov and his colleagues from ARRIAM. The SGECdt is a unique mutant of higher plants

possessing both increased tolerance and accumulation of toxic heavy metals (Tsyganov et al., 2007).

An interesting visit was organised to the electron microscope facility, located at the local University and run by a CNR technician Mr Salvatore Marceddu, who presented introduction about possibilities of electron microscopy to visualize and monitor heavy metals in biological materials, and how this equipment allows understanding details of transport and distribution of heavy metals in plant tissues and plant cell compartments. The facilities available for the CNR group and the model plant such as the SGECdt mutant from the ARRIAM group, represent an excellent combination for further research and for obtaining new and valuable information about mechanisms of metal tolerance/accumulation in plants.

2.5. Preparation of joint publications

All the unpublished data of joint experiments performed during previous years of collaboration were analysed, discussed, grouped together and verified carefully. It has been assumed that the accumulated data are enough for at least two scientific papers. A draft of one paper with a putative title "Growth promoting effects of rhizobacteria on the selected heavy metal tolerant populations of legume species from the Iglesias area of Sardinia" has been prepared during the visit of Dr. Andrey Belimov and now is under final discussion. This paper combines the results about comparison study of different populations of legume plant species in their tolerance to cadmium, lead and zinc as well as their response to inoculation with symbiotic nodule bacteria and associative rhizobacteria containing ACC deaminase. One figure from this paper is presented here (Fig. 5). Draft tables and figures for another paper have been also produced and are being under discussion and improvements. The results of the second paper are mainly related to the accumulation of heavy metals and nutrient elements by various plant species cultivated in metal contaminated mine waste from the Iglesias (SW Sardinia) area.

References

1. Belimov A.A., Hontzeas N., Safronova V.I., Demchinskaya S.V., Piluzza G., Bullitta S., Glick B.R. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol. Biochem. 2005, 37, 241-250.
2. Belimov A.A., Safronova V.I., Sergeyeva T.A., Egorova T.N., Matveyeva V.A, Tsyganov V.E., Borisov A.Y., Tikhonovich I.A., Kluge C., Preisfeld A., Dietz K.-J., Stepanok V.V. Characterisation of plant growth-promoting rhizobacteria isolated from polluted soils and

containing 1-aminocyclopropane-1-carboxylate deaminase. Can. J. Microbiol., 2001, 47, P. 642-652.
3. Belimov A.A., Dodd I.C., Hontzeas N., Theobald J.C., Safronova V.I., Davies W.J. Rhizosphere bacteria containing ACC deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling. New Phytologist, 2009, 181, 413-423.
4. Glick, B.R., Liu, C., Ghosh, S., and Dumbroff, E.B. 1997. Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. Soil Biol. Biochem. **29:** 1233-1239.
5. Glick, B.R., Penrose, D.M., and Li, J. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. **190:** 63-68.
6. Meloni M.C., Piluzza G., Bullitta S., 2000. The potential role of alternative legumes from Asinara island for multiple uses in difficult environments. Cahiers Options méditerranéennes. Vol. 45. 427-430.
7. Pignatti S., 1982. Flora d'Italia. Edagricole, Bologna.
8. Safronova V.I., Chizhevskaya E., Bullitta S., Andronov E., Belimov A. Charles T.C., Lindström K. Presence of a novel 16S-23S rDNA intergenic spacer insert in *Bradyrhizobium canariense.* FEMS Microbiol Letters, 2007, 269, 207-212.
9. Safronova V.I., Piluzza G., Belimov A.A., Bullitta S. Phenotypic and phylogenetic analysis of rhizobia isolated from pasture legumes native of Sardinia and Asinara island. Antonie Van Leeuwenhoek, 2004, 85, 115-127.
10. Tsyganov V.E., Belimov A.A., Borisov A.Y., Safronova V.I., Georgi M., Dietz K.-J., Tikhonovich I.A. A chemically induced new pea (*Pisum sativum* L.) mutant SGECd^t with increased tolerance to and accumulation of cadmium. Ann. Botany, 2007, 99, 227-237.

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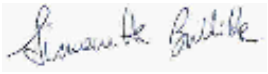
Dr. Simonetta Bullitta 

Fig. 1. Expedition for collecting plant samples.

(A) Legume plant *Lotus cytisoides* growing in saline sand dune in the Stintino area (NW Sardinia). (B) Dr. Andrey Belimov and Mr. Daniele Dettori collect root samples of *L. cytisoides*.

Fig. 2. Mechanism of interactions between plants and rhizobacteria containing ACC deaminase.

See notes in the text.

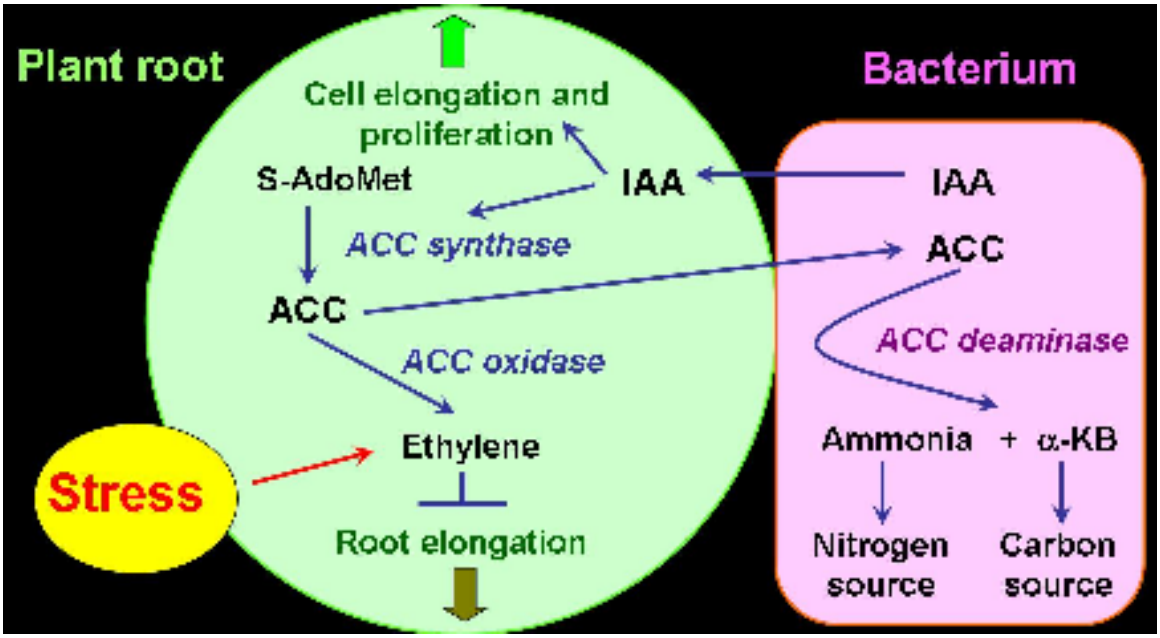


Fig. 3. The first step for isolation of rhizobacteria containing ACC deaminase.

The root homogenates were plated on selective nutrient medium supplemented or not with ACC as a sole nitrogen source. The appeared colonies are putative bacterial ACC utilizers and have to be selected for further purification and testing again for ACC utilization.

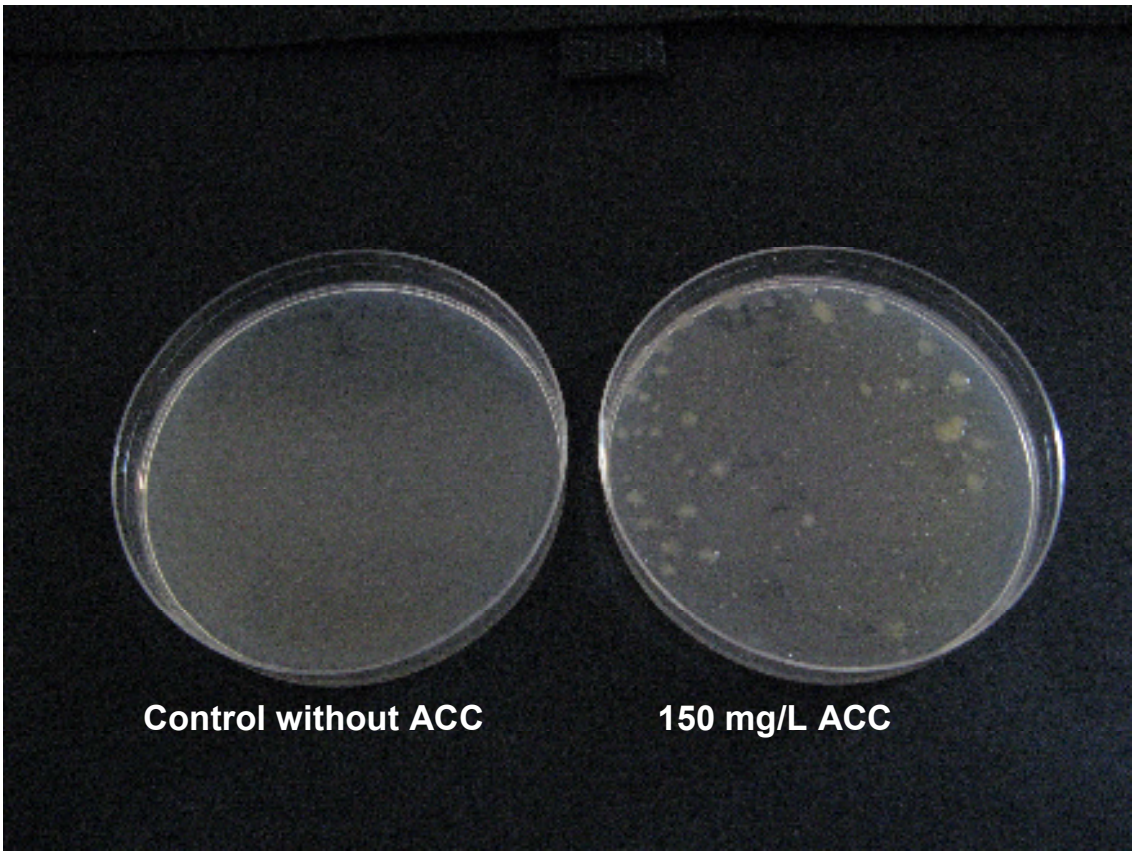


Fig. 4. The cadmium tolerant pea mutant SGECdt.

The plants were grown in nutrient solution supplemented or not with cadmium. (W) wild type pea line SGE; (M) mutant SGECdt.

Fig. 5. Tolerance index of pasture legume species to heavy metals in hydroponic culture. Metal concentrations: (a) 75 µM Zn; (b) 3 µM Cd; (c) 10 µM Pb. Plant species: (Ah) *Astragalus hamosus*; (Le) *Lotus edulis*; (Lo) *Lotus ornithopodioides*; (Mc) *Medicago ciliaris*; (Sm) *Scorpiurus muricatus*. Local plant populations from: (○) Cala d'Oliva-Asinara island; (●) Campo Pisano-Iglesias area. The data are mean, SE (box) and confidential interval (whisker) at $P < 0.05$.