СИСТЕМА ГЕНЕТИЧЕСКОГО ОБРАЗОВАНИЯ

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PROSPECTS FOR THE USE OF MULTI-COMPONENT SYMBIOTIC SYSTEMS OF THE LEGUMES

Legume-Rhizobial Symbiosis (LRS), Arbuscular Mycorrhiza (AM) and associations with Plant Growth-Promoting Bacteria (PGPB) implement nutritional and defensive functions in plant, improve soil fertility, and thus are appropriate to be used for sustainable crop production and soil restoration. Based on synergism and evolutional commonality of the symbioses, we propose a multi-component plant-microbe system with legume plant as a main component. Advances obtained from simultaneous inoculation of legumes with various beneficial microbes are summarized. Basic principles of legume breeding to improve effectiveness of interaction with a complex of the microbes along with problems and prospects for development of multi-microbial inoculants for legumes (and non-legumes) are stated.

❀ **Key words:** Legumes; legume-rhizobia symbiosis; arbuscular mycorrhiza; rhizospheric and endophytic plant growth-promoting bacteria; microbial cooperation; plant breeding; symbiotic effectiveness; multi-component microbial inoculants.

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INTRODUCTION

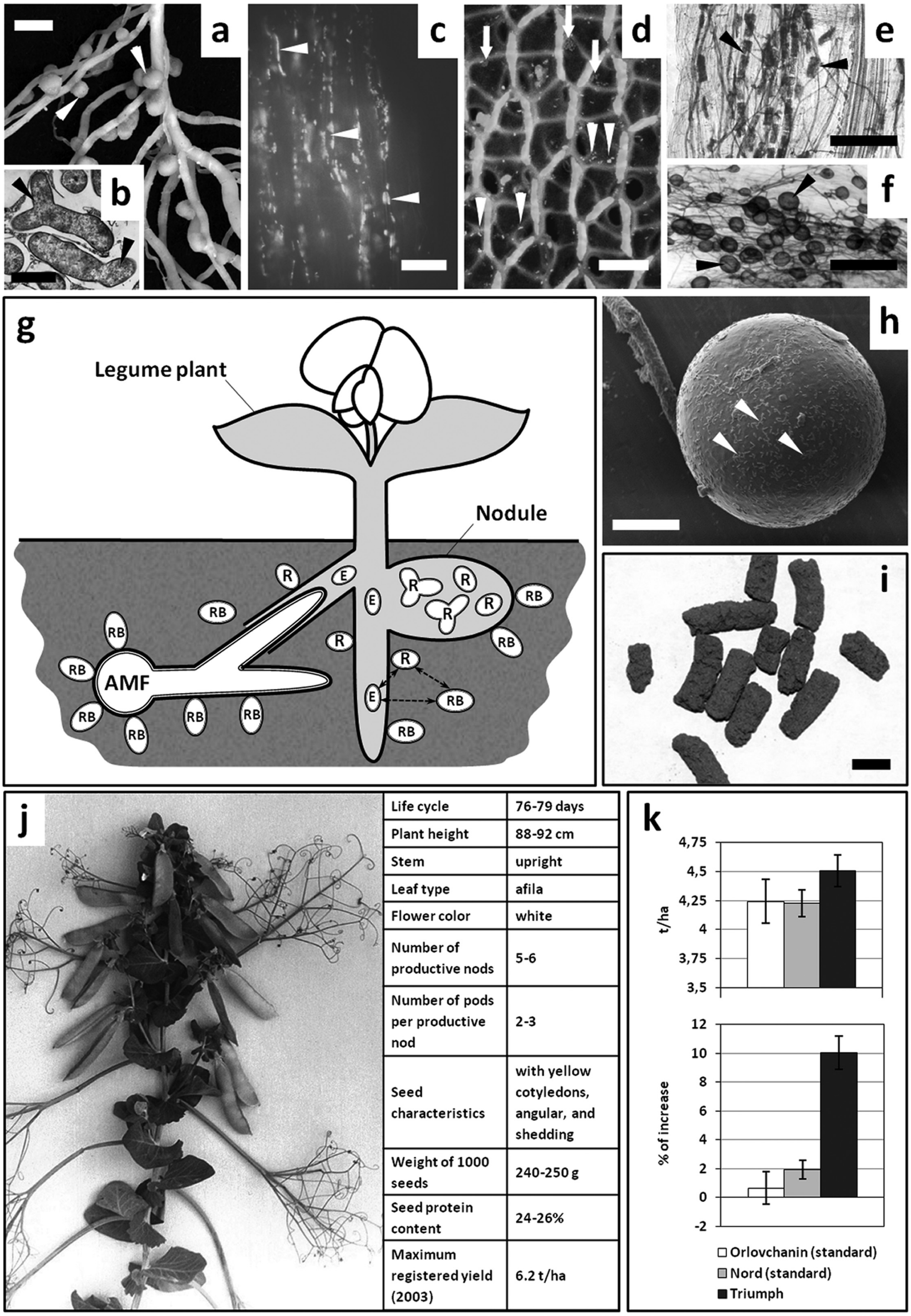
Legumes (*Fabaceae*, Syn. *Leguminosae*), the key components of modern agricultural technologies, represent unique group of plants able to intimately interact with different rhizosphere microorganisms. They form nitrogen-fixing root nodules with rhizobial bacteria (Legume-Rhizobia Symbiosis, LRS) (Dilworth et al., 2008), both rhizospheric and endorhizospheric associations with Plant Growth-Promoting Bacteria (PGPB) (Lugtenberg, 2015; Schulz et al., 2006), and Arbuscular Mycorrhiza (AM) with fungi of phylum *Glomeromycota* (Smith, Read 2008) (Fig. 1 a-f). These symbioses are beneficial for the host plant and its environment as well. They provide plant with nutrients (predominately nitrogen and phosphorus), protect it from biotic and abiotic stresses and improve soil structure and fertility. In return, the microbial partners acquire the carbon from photosynthates and convenient niches for their life and reproduction. The symbioses are controlled genetically by both plant and microbial partner and have evolutional commonality (Hartmann et al., 2009; Lugtenberg, 2015; Oldroyd, Downie, 2008; Ormeño-Orrillo et al., 2013; Provorov, Shtark, 2014; Sessitsch et al., 2002; Shtark et al., 2010, 2012; Smith, Read 2008; Sprent, James, 2007).

The application of microbial inoculants based on the various beneficial soil bacteria and fungi allows improving the crop productivity and decrease the use of mineral fertilizers and pesticides. The vast majority of commercial inoculants available at the moment are based on pure cultures of single microorganism, and only occasionally on their combinations, mainly due to the current procedures for governmental registration and certification of inoculants. Moreover, the use of microbial inoculants in legumes is dominated by an application of rhizobial bacteria to fix atmospheric nitrogen (Gianinazzi, Vosatka, 2004; IJdo et al., 2011; Lugtenberg, 2015; Rai 2006; Wakelin, Ryder, 2004; Xavier et al., 2004). However, there are several objections towards mono-inoculation (see for review: Shtark et al., 2012).

In this review, we propose to combine three aforementioned types of symbiosis in common multi-component plant-microbe systems with legume plants as a basis (Fig. 1 g), and summarize advances of application of combined inoculation of legumes with various beneficial microbes. Basic principles of legume breeding to improve effectiveness of interactions with a complex of beneficial microbes are stated, along with main progress, problems and prospects for development of multi-component microbial inocula for legumes.

MUTUALISTIC ASSOCIATIONS OF THE LEGUMES WITH BACTERIA

The LRS is the formation of special organs called nodules on the plant roots (Fig. 1 a) caused by endosymbiotic bacteria known as rhizobia which subsequently differentiate inside nodules into nitrogen-fixing bacteroids (Fig. 1 b). This symbiosis allows plants to grow without any source of combined nitrogen in the substrate through the acquisition of the ability to fix atmospheric nitrogen.



Life cycle	76-79 days
Plant height	88-92 cm
Stem	upright
Leaf type	afila
Flower color	white
Number of productive nods	5-6
Number of pods per productive nod	2-3
Seed characteristics	with yellow cotyledons, angular, and shedding
Weight of 1000 seeds	240-250 g
Seed protein content	24-26%
Maximum registered yield (2003)	6.2 t/ha

Fig. 1. Biotechnologies of creation of multi-component symbiotic systems in legumes. a–f — Examples of agriculturally and ecologically important plant-microbe symbioses. a — Nitrogen-fixing nodules formed on the root of pea (*Pisum sativum* L.) inoculated with *Rhizobium leguminosarum* bv *viciae*. Scale bar: 5 mm; b — Transmission electron microscopy of ultrathin section of the nodule colonized by rhizobia: arrowheads point to bacteria differentiated into symbiotic forms, the bacteroids. Scale bare: 1 μm; c — Epiluminescence microscopy image of plant growth-promoting rhizobacteria *Pseudomonas fluorescens* RF13H attached to the root surface of tomato (*Solanum lycopersicum* L.) made by using in situ hybridization with universal oligonucleotide probe EUB338 in complex with Cy3 fluorochrome: arrowheads point to bacterial microcolonies. The strain RF13H was isolated as *Sphagnum fallax* moss endophyte (Shcherbakov et al., 2013). Scale bar: 10 μm; d — Confocal laser scanning microscopy image of endophytic bacteria of *S. fallax* gametophytes. *Eubacteria* are visualized in hyaline cells of branch leaves by using fluorescent in situ hybridization with universal (EUB338) and group-specific (BET42a and GAM42a) bacterial probes: arrowheads point to representatives of β-proteobacteria and γ-proteobacteria, arrows — to other representatives of eubacteria. Scale bar: 10 μm. (Images c, d: courtesy of Dr. Andrey Shcherbakov, ARRIAM, St. Petersburg); e, f — Light microscopy of macerated and black ink-stained *P. sativum* roots colonized by fungus *Rhizophagus irregularis* (arbuscular mycorrhiza): arrowheads point to arbuscules (e) or vesicles (young intraradical spores) (f). Scale bare – 50 μm; g — Schematic representation of multi-component symbiotic system of a legume plant: AMF — arbuscular mycorrhizal fungus, R — rhizobia, RB — plant growth-promoting rhizospheric bacteria (PGPR), E — endophytic bacteria. Bidirectional arrows depict probable mutual transitions of bacteria from one symbiotic state to another; h — Scanning electron microscopy of AMF spore with bacteria (marked by arrowheads) attached to its surface. Scale bar: 20 μm. (Courtesy of Sima Kumari, TERI, New Delhi); i — Multi-component microbial fertilizer (MMF) formulated into granules. Scale bar: 5 mm; j — *P. sativum* cv. Triumph and its agriculturally important characteristics; k — Grain yield of cv. Triumph in comparison with productivity standards: overhead — under conditions of conventional technology (metric tons per hectare); below — with application of 500 kg/hectare dose of MMF (percent of increase compared to conventional technology) (Orel district, 2004-2006). Bars represent standard errors.

The nodulation includes well-coordinated genetic programs, the root colonization and the nodule tissue formation, both triggered by successful mutual recognition of the partners (Dilworth et al., 2008; Oldroyd, Downie, 2008; Sessitsch et al., 2002; Sprent, James, 2007). Bacteria able to form nitrogen-fixing nodules on legume roots have extraordinary diversion. They include well-known *Rhizobium*, *Ensifer* (Syn. *Sinorhizobium*), *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, as well as *Methylobacterium*, *Devosia*, *Phyllobacterium*, *Ochrobactrum*, all belonging to α-proteobacteria, some β-proteobacteria (e.g. *Burkholderia, Cupriavidus*) and even γ-proteobacteria (e.g. *Pseudomonas*). Symbiotic genes between unrelated bacteria can be transmitted through horizontal gene transfer, which is common in nature (Ormeño-Orrillo et al., 2013; Provorov, Shtark, 2014; Remigi et al., 2014; Shiraishi et al., 2010; Sprent, James, 2007).

Numerous bacteria among the typical rhizosphere inhabitants dependant on root exudates can exert positive effect on plant growth without formation of specialized symbiotic structures. These bacteria are commonly referred to as PGPR (Plant Growth-Promoting Rhizobacteria) and represent a taxonomically diverse group including *Acetobacter*, *Arthrobacter*, *Azospirillum, Azotobacter, Bacillus, Enterobacter, Pseudomonas, Paenibacillus,* etc. Many of PGPR are able to adhere to root surface and sometimes form biofilm-like structures (Fig. 1 c), though some of them can be detected inside the plant tissues (Bakker et al., 2007; Govindasamy et al., 2010; Hartmann et al., 2009; Ormeño-Orrillo et al., 2013; Yang et al., 2009). The direct impacts of PGPR on the host plant include stimulation of root development via auxin production, associative nitrogen fixation (James 2000; Kennedy et al., 2004; Ormeño-Orrillo et al., 2013), and improvement of phosphorous nutrition by phosphate solubilizing bacteria (Barea et al., 2005; Richardson et al., 2009). The indirect impacts are biocontrol of soilborne pathogenic fungi due to production of antifungal compounds or induction of systemic defence responses in plant tissues (Bakker et al., 2007; Govindasamy et al., 2010; Siddiqui 2006; Vallad, Goodman 2004), and increasing host plant tolerance to abiotic (e.g. drought, heavy metal, salt) stresses through ACC-deaminase, cytokinin and volatile organic compounds production (Bakker et al., 2007; Egamberdieva, Lugtenberg, 2014; Yang et al., 2009).

Usually, interiors of healthy plants (both legumes and non-legumes) grown in the field, forest, or in pot cultures are colonized by populations of non-pathogenic bacteria termed as endophytes (Fig. 1 d). The spectrum of endophytic bacteria covers a wide range of both culturable and non-culturable species. Some endophytes are seed-borne, while for others the colonization mechanisms are still unknown. Many bacterial endophytes demonstrate beneficial features similar to those of PGPR and may assist in phytoremediation. Despite the fact that endophytes possess many virulence factors similar to those of pathogens, the endophytes mainly colonize other niches in plant and have different enzymatic apparatuses (Doty, 2008; James, 2000; López-López et al., 2010; Reinhold-Hurek, Hurek, 2007; Schulz et al., 2006; Shcherbakov et al., 2013; Sturz et al., 2000).

There are speculations about interface between rhizobia and PGPR/endophytic bacteria (Ormeño-Orrillo et al., 2013). Indeed, 'true' rhizobia of α-subclass of proteobacteria are frequent rhizosphere colonizers of a wide range of plants and may also inhabit both leguminous and non-leguminous plants endophytically. These rhizobia may exhibit several plant growth-promoting effects (e.g. auxin production and phosphate solubilization) or suppress pathogens

(Bardin et al., 2004; Hossain, Mårtensson 2008; Huang, Erickson, 2007; López-López et al., 2010; Sessitsch et al., 2002); some *Rhizobium* strains can produce ACC-deaminase (Duan et al., 2009). Probable mutual transitions of bacteria from one symbiotic state to another are shown in Fig. 1 g.

We suggest that the wider collective term, the Plant Growth-Promoting Bacteria (PGPB), is more appropriate because it combines both the beneficial rhizospheric and endophytic bacteria, although the term 'rhizobia' should be left to specifically highlight the bacteria which form nodules in legumes.

ARBUSCULAR MYCORRHIZAL FUNGI AND THEIR COOPERATION WITH BACTERIA

Arbuscular Mycorrhizal Fungi (AMF) of phylum *Glomeromycota* form the most commonly occurring and probably the oldest symbiotic association with more than 80 % of all terrestrial plant species (Smith, Read, 2008). Among the Legumes, *Lupinus* is the only known genus where AM-symbiosis has apparently been lost (Sprent, James 2007). The AMF are obligate biotrophs that colonize plant roots to acquire photosynthates through a special intracellular compartment, the arbuscule (Fig. 1 e), which is a highly branched fungal hypha. The arbuscules remain bound to outer parts of mycelium and act as a single continuum via which the fungus translocates mineral nutrients and water from the soil into the plant root system (Smith, Read, 2008).

The symbiotic association between plant and AMF enhance seed germinating power, improves seedlings establishment and increase the yields and quality of grain, vegetable and fruit crops. The primary function of this association is improving plant nutrition through facilitation of uptake of low-mobile soil nutrients, like the phosphorus. The AM also plays an important role in carbon and nitrogen recycling (Pattinson et al., 2004; Siddiqui et al., 2008; Smith, Read, 2008). In addition, mycorrhized plants have increased resistance to biotic and abiotic stresses (Babikova et al., 2013; Bennett et al., 2009; Bothe, 2012; Campanelli et al., 2013; Currie et al., 2011; Gamalero et al., 2009, 2010; Kempel et al., 2010; Koltai, Kapulnik, 2010; Koricheva et al., 2009; Liu et al., 2007; Siddiqui et al., 2008). The AMF improve soil structure due to release of a glomalin protein by extraradical mycelium (Rillig, 2004; Siddiqu et al., 2008), as well as increase soil fertility (Celik et al., 2004; Mäder et al., 2011). The AMF also play a vital role in interconnecting of roots of different plants . Thanks to the relatively low specificity of AM symbiosis, different plant species (agricultural crops) can benefit from interactions with one (or at least one) fungus in intercropping or in crop rotation systems (Babikova et al., 2013; Smith, Read 2008). Because of their pivotal role in plant and soil ecology, AMF appear to be an essential component in successful revegetation and restoration of degraded soils (Koltai, Kapulnik, 2010; Pattinson et al., 2004).

There is a hypothesis that considers mycorrhizas *per se* as tripartite symbiotic systems: plants — AMF — bacteria (Bonfante, Anca, 2009). The uncultivated and vertically inherited intracellular bacterial symbionts which play an important physiological role in fungal cells are found inside the spores and hyphae of many AMF (Bonfante, Anca, 2009; Naumann et al., 2010). At the same time, it is well known that several bacteria (often PGPB) can form colonies on the surfaces of spores and hypha (Fig. 1 g, h), and many of them induce fungal growth (Artursson et al., 2006; Barea et al., 2005; Hildebrandt et al., 2006). These are so-called "mycorrhiza-helper" bacteria (MHB) (Frey-Klett et al., 2007). The composition of bacterial communities associated with AMF and the properties of individual bacteria depend on the species and physiological state of fungi (Artursson et al., 2006; Scheublin et al., 2010; Toljander et al., 2006). The AMF are able to directly affect these communities, modifying the environment through mycelial exudates and form so-called «mycorrhizosphere» (Barea et al., 2005; Toljander et al., 2007). In addition, the formation of AM affects mineral composition, hormonal balance and carbon distribution in the plant tissues (Barea et al., 2005; Koltai, Kapulnik, 2010; Smith, Read, 2008) and change the composition of root exudates (Hage-Ahmed et al., 2013), thus affecting the rhizosphere microbial community.

The AMF exhibit synergism in the interaction with both indigenous and introduced PGPB involved in biocontrol, nitrogen fixation, mobilization of phosphates, and increasing of plant tolerance to salt and heavy metals (Barea et al., 2005; Behl et al., 2007; Egamberdieva, Lugtenberg 2014; Gamalero et al., 2004, 2008–2010; Hajnal-Jafari et al., 2012; Mäder et al., 2011; Reimann et al., 2008; Siddiqui et al., 2008). The AM symbiosis also enhances formation of nitrogen-fixing nodules in legumes and nitrogen fixation by rhizobia, presumably via stimulation of the host plant mineral nutrition (Barea et al., 2005). In turn, it was revealed that some AMF prefer to colonize legumes and, particularly, old senescent nodules (Scheublin et al., 2004, 2006; Vidal-Dominguez et al., 1994).

ADVANCES IN THE USE OF SIMULTANEOUS INOCULATION OF LEGUMES WITH RHIZOBIA, PGPB, AND AMF

The effect of simultaneous inoculation of legumes by AMF and rhizobia was observed in both pot and field trials, revealing itself in increased productivity and quality of the yield and better protection against pests in comparison with mono-inoculation. That was shown in respect to such legume crops as groundnut (*Arachis hypogaea*) (Ibrahim et al., 1995), chickpea (*Cicer arietinum*) (Lobkov et al., 2011; Siddiqui et al., 2008), soybean (*Glycine max*) (Labutova et al., 2004), lentil (*Lens culinaris*) (Xavier, Germida, 2002), common bean (*Phaseolus vulgaris*) (Naumkina et al., 2012; Parakhin et al., 2008), and pea (*Pisum sativim*) (Borisov et al., 2002, 2004b; Shtark et al., 2006; Jacobi et al., 1999). The synergistic effect of legume plant inoculation with rhizobia and PGPB is also well known. Most often, this effect is associated with stimulation of nodulation and symbiotic nitrogen fixation by PGPB through the production of auxin or ACC-deaminase, and through improvement of plant phosphorous supply (Bakker et al., 2007; Barea et al., 2005; Elkoca et al., 2007; Taurian et al., 2013; Tilak et al., 2006; Tsigie et al., 2011; Yang et al., 2009).

The first experience of triple inoculation of legumes with rhizobia, AMF, and PGPB was described in a pot experiment with albaida (*Anthyllis cytisoides*), a pioneer legume which was dominant in the nutrient-deficient and degraded Mediterranean ecosystem (Requena et al., 1997). Two AMF (native and exotic), two native rhizobial strains, and two PGPB strains (native and exotic) were selected and then used to screen the appropriate double- and triple combinations of microbial inoculants. Many microbial combinations, including triple ones, were shown to improve the plant development, nutrient uptake, nitrogen fixation or root system quality. It was found that simultaneous inoculation with AMF and rhizobia or with individual triple combinations of fungi and bacteria enhance the establishment of albaida and increase soil fertility and quality in terms of soil nitrogen content, organic matter content, soil aggregate hydrostability and enhanced nitrogen transfer from nitrogen-fixing to non-fixing species by means of the natural succession of the plants. These results along with the ones obtained later (Requena et al., 2001) suggest the importance of physiological and genetic adaptation of microbes to the whole environment, promoting the local isolates for inoculum production.

Two later pot experiments involving *Medicago arborea*, a woody legume used for re-vegetation purposes under semiarid conditions (Valdenegro et al., 2001), and *Dalbergia sissoo*, a tropical legume tree (Bisht et al., 2009), supported the existence of selective microbial interactions affecting plant performance. In both experiments, the most efficient microbial treatments involved mycorrhizal inoculation, indicating the AM dependency of those plant species, while inoculation with PGPB was effective only when associated with specific mycorrhizal and rhizobial partners.

As can be seen from two-year field trials conducted in Kampur (India) with dwarf pea (*P. sativum*) and 32 different combinations of rhizobia, AMF, phosphate solubilising bacteria, and fertilizer doses, the strongest effect on pea yield was observed in the variant with triple inoculation against a background of 100 % recommended fertilizers dose (Mishra et al., 2010).

In pot experiments with 99 pea (*P. sativum*) genotypes (Borisov et al., 2002; Jacobi et al., 1999; see for review: Shtark et al., 2010) and in long-term field trials with pea (Borisov et al., 2004b; Shtark et al., 2006), chickpea (*C. arietinum*) (Lobkov et al., 2011), and common bean (*P. vulgaris*) (Naumkina et al., 2011) in the Central region of Russian Federation (Orel district) it has been demonstrated that the plant genotype plays key role in effectiveness of both double inoculation with AMF and rhizobia and multi-microbial inoculation including PGPB. The effect achieved with co-inoculation equaled or exceeded one achieved with mineral fertilizers when using individual pea genotypes (Borisov et al., 2002, 2004b; Shtark et al., 2006). The triple inoculation with rhizobia, AMF and PGPB often yielded a significant increase in pea seed production, as compared to mono-inoculation with rhizobia or combination of AMF with PGPB (Lobkov et al., 2011; Naumkina et al., 2012). Generally, the seed productivity feature was the most responsive to co-inoculation (Borisov et al., 2002, 2004b; Shtark et al., 2006), but it did not correlate with nodule number and nitrogenase activity. In those cases where triple inoculation yielded no increase in pea seed production, it nevertheless resulted in increased seed protein content (Shtark et al., 2006).

Thus, practice shows that in many cases the triple inoculation can provide better results compared with mono- and double inoculation. Nevertheless, the effectiveness of all complex symbiotic systems is influenced by similar factors: microbial strains, plant genotype, and environment (Bisht et al., 2009; Requena et al., 1997; Tilak et al., 2006; Valdenegro et al., 2001). Most researchers emphasize the importance of using local microorganisms' isolates and selection of microbial combinations, taking into account the specific functional compatibility relationships between the components. Finally, since plant genomes are relatively stable in comparison with those of microbes (Provorov, Tikhonovich, 2003; Tikhonovich, Provorov 2007), and contribute significantly to the effectiveness of symbiosis, it is necessary to select plants having high potential for interactions with all groups of beneficial soil microbes.

PLANT BREEDING TO IMPROVE SYMBIOTIC EFFECTIVENESS

Usually, plant breeding to improve symbiotic traits is focused on legumes with respect to their effectiveness of nitrogen fixation (Dilworth et al., 2008; Graham et al., 2004; Herridge and Rose, 2000; Rengel, 2002; Sidorova, 2011; Sidorova et al., 2012, 2014). The breeding approaches include backcross-inbred methods for population development; recurrent selection for enhanced nitrogen fixation; bidirectional selection for specific nodule enzymes; and simple, double, and three-way crossings (Graham et al., 2004). For example, a scheme of recurrent selection was developed involving endemic (primitive) pea (*P. sativum*) forms, cultivars, and both super- and hyper-nodulation pea mutants carrying recessive or dominant mutant alleles of *Sym* genes, respectively. This approach has led to creation of pea genotypes carrying the combination of mutant alleles of *Sym* genes and showing advanced growth and nitrogen fixation characteristics (Sidorova et al., 2012, 2014).

Five crucial steps in developing new legumes for sustainable agriculture were highlighted, which included two steps concerning nodulation: selection or breeding of legumes and their rhizobial genotypes that are adapted to each other and to the target edaphic niche; and assessment of the broader biological implications of both plant and microsymbiont introduction, including "duty of care" issues, such as understanding any biological threat posed by the new material (Dilworth et al., 2008).

A single initial pre-breeding and breeding study has been reported for onion (*Allium* spp.). A high genetic variability for 77 genotypes from an interspecific tri-hybrid population of *A. cepa* × (*A. roylei* × *A. fistulosum*) was demonstrated with respect to their responsiveness to AMF (Galvan et al., 2007), which was high enough to breed crops with the increased symbiotic effectiveness. The possibilities of breeding wheat genotypes responsive to bioinoculants (*Azotobacter* sp. and/or AMF) for sustainable wheat production in semi-arid tropics have also been discussed (Behl et al., 2007).

Our innovative conception of plant breeding determines the need to adjust the internal or external crop's environment to select beneficial soil microbes from natural microbial consortia. This would increase the effectiveness of plant-microbe interactions due to shifting the naturally occurring equilibrium of metabolic integration from microbes to the plant. The selection of plant genotypes should be done using a multi-component microbial inocula consisting of highly effective resident microorganisms (AMF, rhizobia and PGPB), and without mineral fertilizers. Thus, a new trait in legume breeding, an effectiveness of interactions with beneficial soil microbes (EIBSM) has been proposed. It is important that this breeding approach is applicable not only for legumes, but for non-legumes as well. Regarding to grain legumes and forage crops, increases of seed productivity or plant biomass production, respectively, in response to inoculation should be used as the main parameter for an evaluation of plant EIBSM (Shtark et al., 2010; 2012).

In case of pea, a few genotypes with high symbiotic effectiveness were initially identified during the analysis of 99 genotypes (wild-growing or primitive) from the collection of Vavilov Institute of Plant Industry when using a double inoculation with rhizobia and AMF (Borisov et al., 2002). The selected genotypes were involved in breeding programs to create pea cultivars combining high EIBSM and important agricultural traits. The hybrids were selected in special breeding nursery using the inoculation with multi-component microbial inoculant (Shtark et al., 2012; Zhukov et al., 2013). As a result, the first pea cultivar with increased EIBSM in the whole history of legume breeding was created and called Triumph (Fig. 1 j). It arose from five backcrosses and subsequent screening of F_4 hybrid population for high productivity and capacity for supporting various beneficial microbes. The parental genotypes were cv. Classic as a donor of agriculturally important traits, and the primitive genotype K-8274 as a donor of symbiotic effectiveness trait (Borisov et al., 2008; Naumkina et al., 2011). The EIBSM of cv. Triumph is greater than that of both the parental genotypes. It is assumed that the higher symbiotic potential of Triumph may be associated with root exudation of a series of organic acids, which were also among the main components of the root exudates of K-8274 (Kuzmicheva et al., 2014).

The cultivar Triumph is of middle stem height, semi-leafless (Fig. 1 j), and has stable productivity under different climate conditions; it is comparatively resistant to root rots and highly resistant to pea weevil (*Bruchus* (*laria*) *pisorum*). Its productivity is not lower than those of the productivity standards for Orel district using the conventional production technologies and 10 % greater in comparison with the standard cultivars when inoculated with multi-component inoculants (Fig. 1 k). As a result of two-year state trials (2007–2008) the productivity of Triumph was shown to be even greater than that of standard regional cultivars, enabling its recommendation for commercial cultivation in the Central region of Russian Federation (Borisov et al., 2008; Naumkina et al., 2011). Thus, our innovative concept for plant breeding is bearing its first fruits.

Currently, this breeding program is being continued for pea (Shtark et al., 2006; Dr. Tatiana Naumkina, personal communication). In addition, legume crop genotypes with high EIBSM are currently being selected as initial material for further breeding of chickpea (Lobkov et al., 2011), bean (Naumkina et al., 2012), lentil, and soybean (Dr. Tatiana Naumkina, personal communication). Preliminary results indicate that all these crops are genetically polymorphic with respect to EIBSM, and, although the level of the polymorphism is less than that of *P. sativum*, it is still high enough for breeding.

The current development level of molecular biology, genetics, genomics and transcriptomics of both model and crop plants opens up the perspective of reasonable utilization of such genetic polymorphism. Next Generation Sequencing (NGS) technology provides the opportunity to examine the whole diversity of plant genes involved in some biological processes. Of considerable value are symbiotic genes that are not very numerous (about 100 (Borisov et al., 2004a)) but extremely important for proper formation of symbiotic compartments under microbial inoculation.

For model legumes, analysis of association between phenotypic traits (including symbiotic ones) and gene polymorphism is being performed in barrel medic using either Sanger sequencing (de Mita et al., 2011) or NGS (Stanton-Geddes et al., 2013). It was shown using the quantitative trait loci (QTL) mapping in *Medicago truncatula* that two of the symbiotic signaling genes, *NFP* and *DMI3*, are colocalized with two QTL affecting average fruit weight and leaf number, suggesting that natural variation in nodulation genes may potentially influence plant fitness (Gorton et al., 2012). In non-model legumes, such as pea, introduction the NGS technique also facilitated work on SNP marker discovery, linkage map construction and identification of QTLs for agriculturally important traits, for example, enhanced salinity tolerance (Leonforte et al., 2013). Still, marker-assisted selection (MAS) for agronomic traits such as yield, quality, and tolerance to abiotic and biotic stresses is not widely applied in pea due to unavailability of a reference pea genome and the limited number of molecular markers for tagging the agronomically important genes in pea improvement programs (Smýkal et al., 2012).

DEVELOPMENT OF MULTI-COMPONENT MICROBIAL INOCULANTS

The large-scale production of rhizobia- and PGPB-based inoculants is not particularly difficult, since bacteria can be easily cultivated on various nutrient media with the use of fermenters. Once the particular strain for the inoculum has been selected, an industrial standardized process of production can be defined. The materials of carrier can be of various origins: organic, inorganic, or synthesized from specific molecules. Availability and cost are the main factors affecting the choice of both growth media and carrier (Malus et al., 2012; Xavier et al., 2004; Zavalin, 2005). There is increasing interest in clay- and peat-based granular inoculants applied to the soil, and in seed-applied liquid inoculants (Dilworth et al., 2008).

The mass production of AMF inoculum is more problematic, since they are obligate biotrophs (IJdo et al., 2011; Smith, Read, 2008). The substrate-based inoculum is the cheapest one, and thus much more frequently used for large-scale production. It consists of mycorrhized roots of plants cultivated in containers or beds in greenhouse or in field, which are mixed with soil- or sand-based growth substrate (Gianinazzi, Vosatka, 2004; IJdo et al., 2011). One of the major constrains for this method is the necessity of large amounts of inoculum, which means the significant inputs for its transportation to the place of use. Thus, it seems to be expedient to utilize the local manufactures of inoculum (On-farm production) organized in the proximity to final consumers (Douds et al., 2005). In addition, many inoculum producers still develop formulations using different AMF, which are quite often not well characterized in terms of ecological, soil, host or pathogen requirements. The lack of awareness about the purity and effectiveness of AMF inocula leads to cross-contaminations. These, and the lack of quality control for several marketed inocula, are amongst the main reasons for the low acceptance of mycorrhizal technology in agricultural practices (Gianinazzi, Vosatka, 2004).

An alternative technology exploits the genetically modified host roots (Root Organ Culture, ROC) to mass production of viable, healthy, genetically pure and high-grade fungal propagules without any pathogenic contamination under *in vitro* sterile conditions (Declerck et al., 2005; Puri, Adholeya, 2013). The technology was transferred to industries and resulted in the development of commercialized products which are ready available to end users for application. AMF inocula have been formulated as powdered seed coatings, tablets, and suspended in carrier for fluid drilling (Declerck et al., 2005; Douds et al., 2005; IJdo et al., 2011).

A rare example of a developed technology for the production and application of a new multi-microbial inoculant (multi-component microbial fertilizer, MMF) is based on mycorrhized *Sorghum* sp. roots when grown in an unsterile compost-like substrate. The substrate originates from the storage of filtration-washing sediment, a by-product of sugar production from sugar beet, on filtration fields for 20–30 years (Chebotar et al., 2008). Utilization of this type of waste is an important environmental problem. This substrate (pH about 7) consists of soil and lime and possesses its own microbial society including AMF, rhizobia and PGPB (Akhtemova et al., 2010, 2011). Among the cultivable bacteria, the strains with high growth-promoting activity from genera *Bacillus* and *Arthrobacter* are dominating (Akhtemova et al., 2010). When preparing the MMF, the substrate primarily is used for AMF-inoculum production on *Sorghum* sp. under inoculation with highly effective isolates of AMF from the ARRIAM collection. The inoculum then is enriched with a mixture of highly effective strains of rhizobia and PGPB and formulated into granules (Fig. 1 i) or seed coatings (Chebotar et al., 2008). The effectiveness of this approach as well as positive after-effects were demonstrated in year-by-year field trials with legumes and non-legumes (Lobkov et al., 2011; Naumkina et al., 2012; Shtark et al., 2006; Zavalin, Kozhemyakov, 2010).

Being the substrate-based inoculum, the MMF has some difficulties with its quality control, monitoring of the introduced microorganisms in agricultural soils, and especially screening for the presence of pathogenic organisms in the substrate-carrier. At least part of these problems would be probably solved in the near future. Molecular techniques could be used to trace the survival of AMF after introduction into the environment. DNA barcoding based on NGS approach seems to be the most promising for the purpose and is being actively developed (Daniel 2005; Pershina et al., 2013; Stockinger et al., 2010).

In the future similar inocula could obviously be created with ROC as a basis, as AMF could be successfully cultivated *in vitro* together with MHB (Hildebrandt et al., 2006; Scheublin et al., 2010). The microbes possessing both PGPB и MHB features could be isolated from natural environment and then established in ROC of AMF. In order to develop effective AMF-PGPB consortia for creation of complex microbial inoculum as well as continuous co-culture, it is important to study how these combinations would help each other and how these microbes impact the rhizosphere biodiversity.

CONCLUSION

The potential of microbial synergism, which is largely based on the evolutional commonality of the various plant-microbe symbioses, opens up a wide-range of possibilities for the development of biotechnologies to increase both yield and quality of the crop production or re-vegetate desertificed ecosystems with the multi-component legume-microbe systems. The results of numerous experiments on beneficial plant symbioses with AMF, rhizobia and PGPB, including multi-component systems, demonstrate the important role of physiological and genetic adaptations of microorganisms to local environmental conditions. Hence, during the development of the new biotechnologies, it is recommended to use a complex of local multipurpose microbial isolates, adapted to particular environmental conditions.

At the same time, we propose to develop multi-component plant-microbe systems with legume plants as a basis (Fig. 1 g). The effect of the multi-microbial inoculation on plant productivity is more stable when using legume genotypes with high symbiotic effectiveness (EIBSM). High level of genetic polymorphism in legumes, particularly in pea, regarding to EIBSM indicates the possibility and expediency of breeding towards increasing of symbiotic potential. This is supported by successful purposeful creation of the new pea cultivar Triumph demonstrating both high symbiotic efficiency and valuable agricultural features (Fig. 1 j). Cultivars of similar kind could be developed on the basis of many other legumes and non-legumes species, which are already involved in pre-breeding work. This type of selective breeding should be carried on against the background of maximal diversity of beneficial soil microorganisms, which can be provided by multi-microbial inoculation. The total plant biomass and seed weight should be among the main selective traits.

Another significant aspect of this biotechnology is the development of multi-component microbial inoculants formula. Two major courses can be emphasized here; first of them is the elaboration of production technology and application for high-quality substrate-based inoculates with AMF inoculum as the main component. There are several problems associated with application of this technology, such as requirement of large amount of inoculum and difficulties in quality control. Nevertheless, it is such substrate-based inoculum that should be used in breeding of new plant varieties with high EIBSM, for newly-created genotypes could fully realize their adaptive potential. Problem with transporting of large amount of inoculum could be solved by On-farm production, while quality control could be implemented by modern NGS-based molecular genetics approaches. These approaches could also increase the selection rate of high-EIBSM plants. The second direction is the development of pure inoculum based on ROC of AMF. This technology is more complex and expensive, but would be most appropriate to modern requirements for microbial inoculum for their governmental registration and certification. It will also make them more accessible and convenient for use by farmers in the manufacturing of agricultural products based on legumes (as well as non-legume) crops.

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ПЕРСПЕКТИВЫ ИСПОЛЬЗОВАНИЯ МНОГОКОМПОНЕНТНЫХ СИМБИОТИЧЕСКИХ СИСТЕМ БОБОВЫХ

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❀ **РЕЗЮМЕ:** Бобово-ризобиальный симбиоз, арбускулярная микориза и ассоциации с рост-стимулирующими бактериями улучшают минеральное питание растений, защищают их от абиотических и биотических стрессов, повышают плодородие почв и поэтому перспективны для использования в практике адаптивного растениеводства и биоремедиации почв. В обзоре предложены подходы для совмещения этих симбиозов (проявляющих синергизм и связанных общими эволюционными корнями) в единую многокомпонентную растительно-микробную систему на основе бобового растения. Обобщены успехи применения совместной инокуляции бобовых разнообразными полезными микроорганизмами. Сформулированы основные принципы селекции бобовых на повышение эффективности их взаимодействия с комплексом микроорганизмов, а также проблемы и перспективы создания многокомпонентных микробных инокулянтов для бобовых (и небобовых).

❀ **КЛЮЧЕВЫЕ СЛОВА:** бобовые; бобово-ризобиальный симбиоз; арбускулярная микориза; ризосферные и эндофитные рост-стимулирующие бактерии; кооперация микроорганизмов; селекция растений; симбиотическая эффективность; многокомпонентные микробные инокулянты.

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