The alleviation of reforestation challenges by beneficial soil microorganisms

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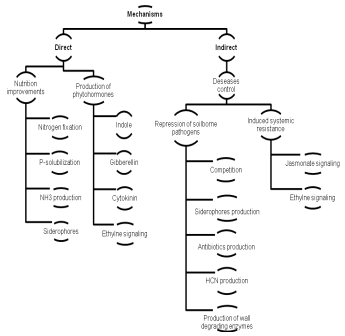

ARTICLE INFO

Citation:
Karličić V, Golubović Ćurguz V, Raičević V (2016) The alleviation of reforestation challenges by beneficial soil microorganisms. Reforesta 1:238-260.
DOI: http://dx.doi.org/10.21750/REFOR.1.12.12

Editor: Vladan Ivetić, Serbia
Received: 2016-02-07
Accepted: 2016-05-10
Published: 2016-05-25

This article is Chapter 11 of establishing issue of Reforesta Journal, edited in form of Thematic Proceedings, by Vladan Ivetić and Steven Grossnickle.

Abstract

Surface mining causes major destruction of natural landscapes and ecosystems. The most fertile, surface soil layer is lost permanently, together with vegetation, wildlife, and micro flora. Post-mining areas are characterized with diverse edaphic, topographic, hydrographic conditions, which complicate land restoration. Successful establishment of forest ecosystems on such land depends mostly on selection of tree species. The chosen plants must be capable of tolerating a wide range of acidity, fertility, moisture, and have potential to ameliorate such substrates for more demanding species. But, reforestation of heavily damaged ecosystems, such as post-mining areas, demands a new approach in seedlings production. This new approach takes into account specific requirements of habitat and integrates them into "targeted production of planting material". A good strategy for successful reforestation of post-mining areas is the input of organic matter (compost, mulch). Also, current knowledge and experiences emphasize the potential of beneficial microorganisms such as, mycorrhizal fungi (MF) and plant growth promoting rhizobacteria (PGPR). The majority of studies that deal with beneficial interactions between trees and microorganisms are focused on the mycorrhiza, while plant growth promoting rhizobacteria are less present in silviculture. In this study, the focus is on the reforestation challenges of two mining basins, Majdanpek and Kolubara and suggests beneficial microorganisms as potential solution. The study presents results of several years' researches on plant response to the presence of mycorrhizal fungi and PGPR. The substrates used for plant growth were Majdanpek and Kolubara mine deposals. Mycorrhizal seedlings were grown in Majdanpek mine deposal, and at the end of the experiment they had 30% higher biomass in comparison to control (seedlings without mycorrhiza). Seedlings linked with fungi had a higher survival rate. Deposals from Kolubara Mining Basin were used as a substrate for seedlings inoculated with PGPR. In the first experiment, Scots pine and Norway spruce were inoculated with *Azotobacter chroococcum, Bacillus megaterium, B. circulans, B. licheniformis, B. pumilus, B. amyloliquefaciens.* Inoculation resulted with higher biomass production (Scots pine 43%, Norway spruce 34%). Similar results were obtained in the second experiment where Scots pine and black locust were inoculated with *Bacillus licheniformis, Aeromonas hydrophila, Pseudomonas putida and Burkholderia cepacia.* Both species had higher biomass (around 20%) in comparison to un-inoculated control. The results confirmed the fact that early establishment and successful growth of vegetation on devastated areas depends on the presence and activity of soil microbes. Microorganisms as a "nature's solution" pose the potential to alleviate reforestation challenges of anthropogenic devastated landscapes. Their presence and activity is crucial for ecosystem stability. In areas with compromised balance, their introduction is justified action for achieving the goal of long term ecosystem sustainability.

Keywords

Post-mining Landscapes, Reforestation, Mycorrhizal Fungi, Plant Growth Promoting Rhizobacteria

Contents

1 Introduction

In the Republic of Serbia, 55 producers of forest seedlings with a total of 87 nurseries are registered (Directorate of Forests, Ministry of Agriculture and the Environmental Protection, the Republic of Serbia). Production in forest nurseries is oriented towards: (a) plant material for reforestation and/or reclamation programs, (b) plant material for the domestic market, i.e. for unknown buyers, and (c) plant material for export (Isajev et al. 2010).

Production of the plant material for the reforestation programs is based on the attitude that high-quality seedling material is a key factor for successful reforestation. Morphological and physiological characteristics of seedling material predispose them to survive the expected range of environmental conditions (Davis and Jacobs 2005). Production of such seedlings does not consider specific requirements of the site. This fact highlights the need for moving traditional seedling production towards *targeted production of planting material.*

The traditional concept is simple, linear, and based on the mass production of uniform quality seedlings (Isajev et al. 2006). Production of targeted seedlings is based on special cultural treatments that prepare them to survive and grow in precise habitats and meet specific objectives of the site (Rose et al. 1990). Unfavorable effects of site-specific factors can be mitigated through specific-purpose production of the conditioned planting stock (Isajev et al. 2010). The basic concept of "target plant materials" is reflected through the fact that seedlings need to be characterized by morphological/physiological characteristics and by the performances of future habitats (Landis 2011).

Areas devastated by mineral resources exploitation represent the places of specific interest for reclamation and reforestation projects. Those sites are characterized by major destruction of natural landscapes (Rakić et al. 2011). At the end of the mining activity, despite the scale of destruction, damaged land needs to be incorporated into surroundings with simultaneous productive ecosystems recovery. Technical re-cultivation creates a new image of space and prepares the surface for the biological re-cultivation whose main goal is bringing back vegetation. This goal is usually achieved through reforestation.

The success of biological re-cultivation greatly depends on plant material quality. But, very often, quality plant material fails to adapt to new environments. The major reason for poor performances of high quality seedling material is the equal approach applied in their production and neglected habitat conditions (Golubović Ćurguz et al. 2012). Under these circumstances, the success of reforestation is uncertain, regardless of the choice of species and their quality (Veselinović et al. 2010). Nursery production of seedlings intended for biological re-cultivation of post-mining areas must take into account unfavorable conditions of the site and focus on the "targeted production".

In the last few years, inoculation with beneficial microorganisms emerged as a new technique incorporated into seedling material production. Literature data emphasize that soil amendment with beneficial microorganisms increase plant survival, seedling quality, and represents an alternative to mineral fertilizers (Chanway 1997; Probanza et al. 2001; Dominguez-Nuñez et al. 2015). The main stock of beneficial microorganisms is rhizosphere, the soil layer closest to the roots. During seed germination and seedling growth, plants interact with a wide range of microorganisms in this zone (Vrbničanin et al. 2008). Connections established in rhizosphere have major influences on newly formed vegetation forms and their future development dynamic. The aim of this paper was to present new concepts in plant material production for the specific purposes such as re-cultivation of post mining areas. Our main goal was to emphasize the power of microorganisms as crucial factors for seedlings accommodation. Also, we tried to emphasize the importance of seedlings final destination characteristics in the processes of their production. The Majdanpek and Kolubara Mine Basins (Serbia) were used as examples. Through our results, we tried to suggest that combination of site-specific conditions with inoculation might represent the most promising way for solving reforestation challenges.

2 Reforestation challenges of areas devastated by mining activity

2.1 Locations of interest: Majdanpek Mine and coal mine Kolubara

Surface mining causes major destruction of natural landscapes, accompanied with the removal of entire vegetation, followed by wildlife, microflora devastation, and permanent loss of the most fertile, surface soil layer (Rakić et al. 2011). The final phase of mining activity is landscape restoration whose purpose is to reintegrate damaged land into surroundings and recover productive ecosystems. Generally, it is not possible to recreate previous ecosystems, but new land use must be incorporated in wider area through rebuilding agricultural land, forests, parks, recreational zones, industrial complexes, residential buildings etc.

The crucial problem connected with re-cultivation of post mining areas is lack of a fertile soil layer, low content of humus, variability of substrate mechanical characteristics (Golubović Ćurguz et al. 2010a), and a wide range of acidity (Rakić et al. 2011). Post-mining areas are characterized with diverse edaphic, topographic, hydrographic conditions (Maiti 2007; Tischew et al. 2008), and low microbial diversity and activity (Golić et al. 2006; Raičević et al. 2006). All these factors limit the group of suitable tree species to those capable of tolerating different environmental conditions (acidity, fertility, moisture).

One of our places of interest was Majdanpek Copper Mine (Bor District, Serbia). Majdanpek Mine was known back in the Roman times, and the surface exploitation began in 1958. It falls into the category of mines with big and deep open pits. During the time, the excavation and overburden deposits changed the original forest hilly-mountain terrain into a completely different form of terrain. As a result of the mining activities, more than 800ha had been degraded. Table 1 shows the chemical properties and heavy metal content (Cu, Pb, Ni and Cd) of deposols sampled from five different, randomly selected localities, while table 2 shows microbial activity of those localities.

Table 1. Chemical Properties of deposol.

Localities and depth	pH		Total		P_2O_5	K_2O	Heavy metals			
	H_2O	KCl	humus %	N %	mg/100g	mg/100g	Cu	Pb	Cd	Ni
							ppm			
A (0-10 cm)	2.10	1.90	4.80	0.07	1.8	1.74	20.3	7.9	0.1	9.3
B (0-10 cm)	2.30	2.00	0.97	0.09	0.3	1.97	90.0	8.1	0.2	0.9
C (0-10 cm)	2.80	2.00	0.86	0.08	0.4	2.58	32.8	7.3	0.0	0.7
D (0-10 cm)	2.30	1.90	0.88	0.17	14.6	3.60	37.3	6.5	0.0	11.3
E (0-10 cm)	3.30	2.40	0.97	0.14	0.3	5.20	55.5	8.1	0.0	1.0

The deposols of Majdanpek Copper Mine are characterised by the high variability which is the main characteristic of the anthropogenically degraded soils (Dražić 2002). Except the A locality, all others possess very low humus content. The total nitrogen content is low at all studied localities, which points to a wide C/N ratio and the poor conditions for mineralization of organic nitrogen and its occurrence in accessible forms. The easily accessible forms of potassium and phosphorus are extremely deficient in deposals. A good phosphorus supply is reported only at locality D. These substrates are also characterised by the high concentration of toxic metals. Heavy metals can be limiting factors in re-cultivation processes. The results show that the greatest Cu, Pb and Cd content are present in sample B. Further studies were conducted with deposals from this location. The accessibility of heavy metals for plants depends upon the soil acidity. The reduction of pH values increases the solubility of cadmium, zinc. The measurements of pH values characterized all samples as very acid. As a result of all these factors, there is a poor/no presence of plants at these localities.

Microbiological analysis of deposols showed extremely weak activity (Tab. 2). Bacteria are the most abundant group of soil microorganisms and their usual number is between 10^6 - 10^9 CFU g^{-1} (Vieira and Nahas 2005). In Majdanpek Mine deposal countered number was ten times lower. The usual number of soil fungi is between 10^4 – 10^6 CFU g^{-1} (Zuberer and Wollum 2004) but in the case of those deposals, no fungi presence was recorded.

Table 2. Microbiological Properties of deposol (CFU g^{-1}).

Locations and depth	Total bacteria x 10^5	Total free diazotrophs x 10^5	Total fungi x 10^3
A (0-10 cm)	2.23	1.67	/
B (0-10 cm)	3.23	2.53	/
C (0-10 cm)	4.14	2.28	/
D (0-10 cm)	3.39	2.74	/
E (0-10 cm)	5.15	1.80	/

This characteristic may be an aggravating factor for future recultivation activities. Still, little is known about the functional diversity and metabolic abilities of microbial communities in developing mine soils (Chodak et al. 2009). Microorganisms are considered key factors that affect soil quality, aggregate formation, plants nutrition and health, aboveground ecosystems, their recovery and stability (Kirk et al. 2004; Raičević et al. 2010). On the other hand, the presence of vegetation encourages microbial abundance, proliferation and activity (Petričević et al 2012; Radić et al. 2013; Avera 2014). Lack of vegetation, as a consequence of unfavourable chemical characteristics of deposols, is one of the reasons of poor microbial activity.

The second place of interest was the biggest open pit mine in Serbia, Kolubara Lignite Mine (Lazarevac District, Serbia). Kolubara Mine started open-cut coal mining in 1952. The region of the Kolubara Basin covers an area of 600 km^2. Open-cast coal mining has completely changed the topography of this area. The hilly terrain in the eastern part of the Basin was leveled and flattened landforms were formed by the deposited material. The flat areas of Tamnava were raised, and the elevated plateau was formed. The recultivation processes in the Kolubara Basin are expanding each year. So far 971 ha were recultivated, with 1,942,000 seedlings planted (Veselinović et al. 2010). On the other hand, mining activity is very dynamic and re-cultivated areas are often re-involved in the lignite exploitation (Živanović Miljković and Džunić 2013). At this point, overburdened waste dumps cover 3,395 ha that needs to be re-cultivated (Jakovljević et al. 2015).

Table 3 shows the chemical properties of Kolubara mine deposal. The basic chemical analysis revealed that the major defect of substrate that covers this huge area is lack of organic matter.

Table 3. Chemical Characteristics of Kolubara Mine deposol.

Location and depth	pH		$CaCO_3$ %	total		P_2O_5	K_2O
	H_2O	KCl		humus %	N %	mg/100g	mg/100g
A (0-10 cm)	7.25	6.2	0.1	0.15	0.018	0.4	10.4

The nitrogen content, accessible forms of phosphorus and potassium are deficient in this deposal. On the other hand, pH value is suitable for most plant species, spontaneous colonization by plants is obvious. Literature data shows that some fragments of Kolubara Basin have elevated concentrations of cobalt, nickel and chromium (Danilović et al. 2013).

The microbiological characteristics of Kolubara deposal is presented in table 4 and those analysis showed higher activity compared to the deposal from Majdanpek Mine. Those results are expected, since normal pH value and presence of vegetation suggested that processes of spontaneous successions are in progress. The number of present bacteria and fungi corresponding to numbers countered in poor soils.

Table 4. Microbiological Properties of Kolubara Mine Deposol (CFU g^{-1}).

Location and depth	Total bacteria x10^6	Total free diazotrophs x10^4	Total fungi x10^4
A (0-10 cm)	4,1	0,24	1

So far, re-cultivation activities of this area employed tree species such as black pine, Scots pine, European larch, European ash, small-leaved Lime, black locust (Veselinović and Golubović-Ćurguz 2001; Rakić et al. 2011). Those species tolerate a variety of soils and grow predominantly on nutrient deficient substrates.

3 The alleviation of reforestation challenges

The levels of seedling survival after replanting is the main problem that forest nurseries are facing all over the world. Poor seedling survival and inadequate early growth performance lower the chances of successful forest regeneration. The prevailing attitude states: "The quality of the seedlings produced in the nurseries determines the future success (Puente et al. 2010), especially in anthropogenic impacted environments." Lately, entering inoculation with beneficial microorganisms into standard nurseries procedures emerged as the possible solution for the alleviation of reforestation challenges (Puente et al. 2010; Pereira and Castro 2014).

3.1 Natures solution: Beneficial soil microorganisms (BM)

Current knowledge and experiences accentuate mycorrhizal fungi (MF) and plant growth promoting rhizobacteria (PGPR) as promising solutions from nature. Soil amendment with ectomycorrhizal fungi and plant-growth-promoting rhizobacteria increase plant survival and seedling quality, especially in substrates with low microbial activity (Chanway 1997; Probanza et al. 2001). Beneficial microorganisms are well known, due to their ability to colonize and elicit stimulant effect on plant growth and health. Also, they represent an alternative to conventional fertilizers and contribute to the conservation of soil biodiversity (Dominguez-Nuñez et al. 2015). Soil microorganisms are getting more and more popular as bioremediation agents as well (Kavamura et al. 2008, Lalević et al. 2012; Jovičić Petrović et al. 2014; Atanasković et al. 2015).

All over the world, forestry nurseries apply mycorrhization as a way to obtain better quality seedlings. The mycorrhizal seedlings are mainly intended for the reforestation of the degraded terrains and re-cultivation of soils damaged by surface exploitation of natural resources (Marx et al. 2002). Nevertheless, in spite of the fact that the production of forest mycorrhizal seedlings is widespread in many American and European countries (Castellano and Molina 1989) this type of seedling material isn't produced in Serbia.

Studies on trees - microorganism's beneficial interactions are mainly focused on the mycorrhiza, while PGPR are less present in silviculture. On the other hand, actual research shows the ability of PGPR to improve plant growth, survival of outplanted seedlings and stimulate plant networking with mycorrhizal fungi and nitrogen-fixing bacteria (Barriuso et al. 2008; Nadeem et al. 2014). Inoculation with both mycorrhizal fungi and PGPR, improves the physical, chemical, and biological soil properties and increase substrate fertility (Caravaca et al. 2003; Graf and Frei 2013). On the other hand, worldwide growing struggles to reduce the use of mineral fertilizers and chemicals resulted with strong growing market of bacterial inoculants (Tilak et al. 2005). That affected nursery practice in Serbia, even though the usage is still on pretty modest levels (Raičević et al. 2006).

3.2 Beneficial soil microorganisms - Mycorrhizal fungi (MF)

Mycorrhiza is a symbiotic association situated in the root zone, sharing the space with numerous pathogenic and saprophytic microorganisms (Priha 1999). This association brings benefits to cohabitants, plant and fungi. The majority of mycorrhizal fungi belong to basidiomycetes (*Suillus* spp., *Amanita* spp., *Lactarius* spp., *Pisolithus* spp., *Rhizopogon* spp.) while some of them are ascomycetes (*Cenococcum* spp., *Elaphomyces* spp., *Tuber* spp.) (Dahm 2005). The fact that 80% of plant species (Lingua et al. 2013) form symbiotic relations with mycorrhizal fungi, confirms the importance and necessity of its presence. Positive effects are results of improved plant mineral nutrition (Smith and Read 2008), synthesis of secondary metabolites (Baslam et al. 2013), connections and nutrient transfer between plants (Izumi et al. 2006; Rigamonte et al. 2010) and soil particles aggregation (Graf and Frei 2013). The mycorrhizal fungi use different root exudates (Kieliszewska-Rokicka et al. 2000). In return they improve the plant nutrient supply (Colpaert et al. 1999), increase root mass (Dahm 2005; Baum et al. 2006), and protect root systems from pathogens (Inglis and Kawchuk 2002). In addition, they enable the absorption and translocation of water in plants (Plamboeck et al. 2007), protect plants from the drought (Rudawska et al. 2000; Ortas 2003), temperature extremes (Karen 1997), and mitigate the negative influences of heavy metals (Rudawska et al. 2000).

After replanting on the final location, successful growth of plants is highly influenced by the establishment of mutualistic relations with MF (Golubović Ćurguz et al. 2010b). Often, seedlings used for reforestation or re-cultivation of devastated areas are not properly prepared for unfriendly climatic and edaphic conditions. Usually, this leads towards low survival rates. Often, the presence of mycorrhiza is the only precondition for survival and successful growth in unfriendly environments (Rudawska et al. 2000).

The best moment for seedlings mycorrhization is during their nursery time. The soil influences the presence of the mycorrhizzal fungi by its physical and chemical charcteristics and, mainly, by the increased concentrations of heavy metals (Lux et al. 2001; Jacob et al. 2001; Puhe 2003). Higher concentrations of heavy metals are toxic to all organisms and change the number, composition, and diversity of soil microbial populations (Rudawska et al. 2000).

The presence of plants inoculated by the mycorrhizal fungi is necessary in the vicinity of the mines which are characterized with low natural infection potential. Inoculation of plant species with mycorrhizal fungi make their root system more adaptable to extreme soil conditions. This leads towards increased nutrient adoption and better plant growth (Totola and Borges 2000) in comparison to non-mycorrhizal seedlings (Huang et al. 2000). Previous experiences show successfully recultivation of mining areas by employing mycorrhizal seedlings in reforestation projects (Marx et al. 2002; Münzenberger et al. 2004).

3.3 Beneficial soil microorganisms - Plant Growth-Promoting Rhizobacteria (PGPR)

The main characteristic of this group of soil bacteria is its ability to accelerate plant growth. This bacterium mostly populates rhizosphere, root surface and root inner tissues, while bulk soil is much less inhabited (Hayat et al. 2010). Numerous mechanisms are involved in plant growth promotion and the stabilization of plant´s physiological state and health. Some of them are well known, while others are not completely identified. Mechanisms of PGPR action can be divided into direct and indirect group; some of the most studied are presented in Figure 1. Direct mechanisms are referred to plant nutrition and hormonal status, while indirect mechanisms manage plant-pathogen interactions.

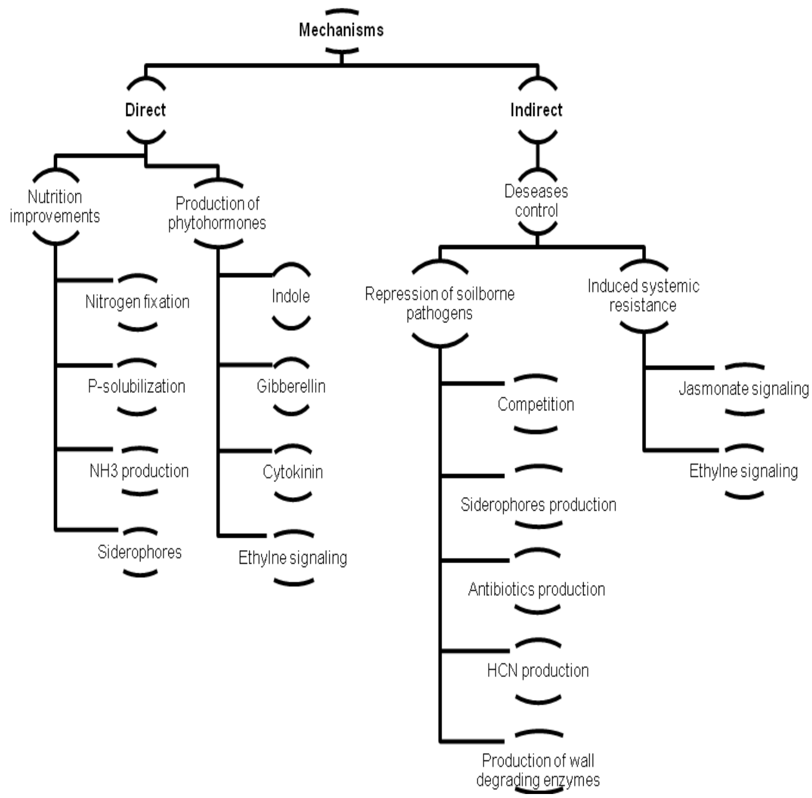

Figure 1. PGPB mechanisms.

Based on the mechanisms, PGPR are often referred as bio-fertilizers (direct mechanisms) or bio-control agents (indirect mechanisms). There are a lot of products on the market specified for plant promotion or pathogen suppression. Commercial products are usually based on single strain formulations (Figueiredo et al. 2010). New tendencies emphasize the ecological advantage and synergistic effects connected with inoculums consisted of several strains (Raičević at al. 2010). This is the direction that bio-inoculants production is taking more and more nowadays.

The first place in bio-inoculants production is reserved for symbiotic bacteria (*Rhizobium, Bradyrhizobium, Mesorhizobium*) and several non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillum, Azomonas*) (Ahemad and Kibret 2014). These bacteria also have the ability to exert a positive influence on plants exposed to various environmental stresses, such as salinity (Qurashi and Sabri 2012), heavy metals (Hao et al. 2012), and xenobiotic (Ahemad and Khan 2012). There is also an option of using a co-inoculation approach; literature confirms that mycorrhizal fungi used with PGPR may act synergistically (Gamalero et al. 2010). An important amount of soil microorganisms are known as mycorrhization helper bacteria (MHB), which are capable to stimulate hyphal growth and assist mycorrhization (Kurth et al. 2013). Some of the main MHBs are members of *Agrobacterium, Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella, Rhizobium, Bacillus, Brevibacillus, Paenibacillus*, *Rhodococcus, Streptomyces, Arthrobacter* genera (Frey-Klett et al. 2007). Members of mentioned genera are also well known as plant-growth-promoting rhizobacteria (Shilev et al. 2007). Distinctions between those two groups are difficult to make.

3.3.1 Application of PGPB in forestry

Compared to agriculture, PGPR application in forestry is much less studied. But certain interest has been present for several decades. Literature data confirm that inoculation conducted in a laboratory, growth chamber, green house, or field result with significant plant growth enhancement (Chanway et al. 2000; Probanza et al. 2002; Karlidag et al. 2007). PGPR application results with higher biomass production, higher survival rate, shoot length, shoot surface area, and shoot and root dry weight, root growth and morphology, germination rates, leaf area, chlorophyll content, magnesium, nitrogen and protein content, tolerance to drought, salt, heavy metals stresses (Jing et al. 2007; Puente et a. 2010). Increases in shoot and root length and weight are commonly reported reactions to PGPB inoculations (Vessey 2003; Gujaničić et al. 2012). Effects of PGPR bacteria are mainly assayed on *Pinus, Picea, Tsuga*, *Pseudotsuga* and *Eucalyptus* (Chanway 1997; Mafia et al. 2009). Some of the most recent studies are presented in Table 5.

Unfortunately, pesticides and mineral fertilizers are part of standard protocols in modern nurseries. Over the years, constant application of chemicals resulted to a reduction of BM, soil contamination (Smith and Read 2008; Domingurz- Nuñez et al. 2014), and negative influences on the environment (Rodrigez et al. 2006). On the other hand, beneficial microorganisms represent an “environmental friendly” alternative to mineral fertilizers and pesticides used in nurseries (Chanway 1997; Dominguez-Nuñez et al. 2014). PGPR are already recognized as an effective tool in moving modern agriculture to a more sustainable manner. Those microorganisms can use that potential in forestry and environmental restoration activities (Lucy et al. 2004). Their application reduces the need for chemical inputs, resulting in ecological and economic benefits.

Previous experiences emphasize the effectiveness of PGPR in nutrient deficient substrates, compared to those were nutrients are more available (Egamberdiyeva 2007). In such substrates, bacteria take the role of nutrient providers (Grobelak et al. 2015).

Large-scale utilization of PGPR is limited by the fact that their effects are under great influence of biotic factors. Often, the strains that show pretty satisfying results in laboratory conditions show no affects in field. The field is an unforeseeable environment whose properties can influence PGPR effectiveness. After being applyed to nature, PGPR are facing better-acclimated domestic micro-flora (Rincón et al. 2008). The effectiveness of their application is dependent on the ability to survive highly competitive rhizosphere ambience, colonize root surrounding and root inner tissues (Lugtenberg et al. 1999; Whipps 2001).

Table 5. Examples of PGP Bacteria Tested on Tree Species.

PGPR	Plant	Benefits	Authors
***Bacillus* sp.; *Microbacterium* sp.**	*Malus domestica L.*	growth, yield, Ca, K, Fe, Cu, Mn and Zn leaf content	Karlidag et al. 2007
Pseudomonas fluorescens	*Pinus halepensis* Mill.; *Quercus coccifera* L.	growth	Rincón et al. 2008
Pseudomonas* sp.; *Stenotrophomonas maltophilia; Bacillus subtilis	*Eucalyptus* sp.	seed germination, height, root and shoot dry weights	Mafia et al. 2009
Burkholderia cepacia	*Morus alba* L.	pathogen inhibition, growth	Ji et al. 2010
Azotospirillum brasilense	*Eucalyptus globules* Labill.	seed germination, early seedling growth, root biomass	Puente et al. 2010
Agrobacterium tumefaciens	*Robinia pseudoacacia* L.	growth, biomass	Hao et al. 2012
Azotobacter chroococcum; Bacillus megaterium;B. circulans; B. licheniformis; B. pumilus; B. amyloliquefaciens	*Pinus sylvestris* L.; *Picea abies* L. Karst	growth	Gujaničić et al. 2012
Paenibacillus polymyxa	*Thuja plicata* Donn.	growth, foliar N content	Anand et al. 2013a
Paenibacillus polymyxa	*Pinus contorta var. latifolia* (Dougl.) Engelm.	growth, lower mortality, foliar N content	Anand et al. 2013b
***Azotobacter chroococcum; Streptomyces* sp.**	*Ulmus pumila* L.; *Robinia pseudoacacia* L.; *Acer dasycarpum* Ehrh.	growth	Jafari et al. 2014
Azotobacter chroococcum; Azospirillum lipoferum; Pseudomonas fluorescens; Bacillus subtilis	*Crataegus pseudoheterophylla* Pojark.	germination rate	Fatemeh et al. 2014
Bacillus licheniformis*; *Aeromonas hydrophila*; *Pseudomonas putida*; *Burkholderia cepacia	*Robinia pseudoacacia* L.; *Pinus sylvestris* L.	growth	Karličić et al. 2015
***Mesorhizobium* sp.**	*Betula pubescens* Ehrh.	growth, root biomass	Sousa et al. 2015

4 Experiences from the scene

4.1 Majdanpek Mine

Mine sites are extremely inhospitable for plant growth. The presence of symbiotic relationships is the defining factor between life and death (Tredici 2008). We already mentioned that the best moment for plant inoculation with mycorrhizal fungi is nursery time. During this period, plants are constantly observed and the environmental conditions are controlled to some extent. In the end, seedlings undergo selection and only those with formed mycorrhiza can be used for reforestation.

The goal of this phase of our work was the production of healthy mycorrhizal seedlings. Selection of seedlings capable to adjust to Majdanpek Mine soil conditions was conducted through the introduction of modified deposal from B locality in seedling production. Keeping in mind the chemical characteristics of this particular deposal (Tab. 1), especially heavy metals concentrations, heavy metals tolerance of mycorrhizal fungi was assessed in pre-test.

4.1.1 The influence of heavy metals on mycorrhizal fungi growth (the first pre-test) (Golubović Ćurguz et al. 2010a)

Suillus granulatus, Suillus luteus, Suillus bovinus, *Hebeloma* spp., *Paxillus involutus* and *Amanita muscaria* reactions to the presence of zinc, copper, lead and cadmium was assessed. Malt Extract Agar (MEA) medium amended with 0, 3, 33 and 100 ppm of these ions in the form of $ZnSO_4$ x $7H_2O$, $CuSO_4$ x $5H_2O$, $Pb(COOH)_2$, $CdSO_4$ x $8H_2O$ was prepared and autoclaved. MEA plates without additional heavy metals were used as control. Mycelia discs of 1cm in diameter were cut and placed on the center of the control and heavy metals-enriched plates. Three replicate plates were used per treatment. The radial growth of mycelium was monitored by the measurement of two cross-sectional diameters, and the obtained values were compared with the growth on the control plates. The effects of heavy metals presence on the growth of fungus species are presented in the in Figures 2-5. The heavy metals influenced the growth rate of all fungi, depending on the type of metals and their concentration.

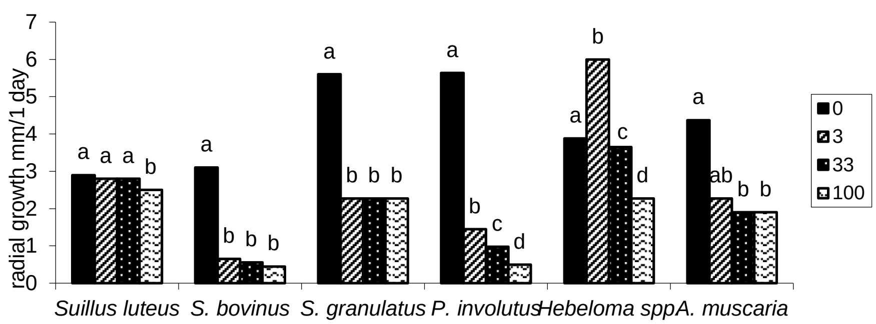

Figure 2. The rate of growth (mm/1day) of mycelium of the mycorrhizal fungi on MEA medium with the different concentration of zinc.

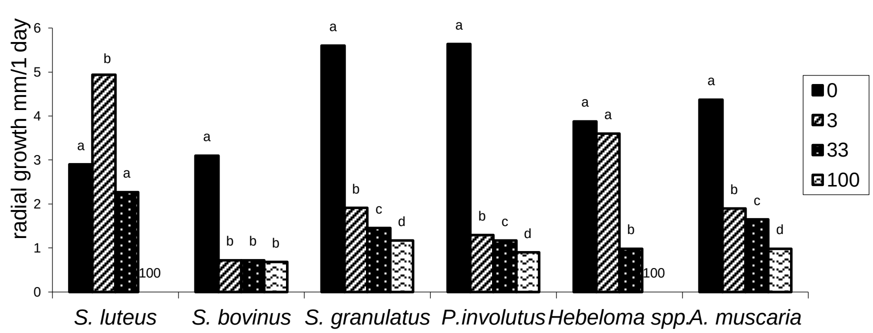

Figure 3. The rate of growth (mm/1day) of mycelium of micorrhizal fungi on MEA with different concentrations of copper.

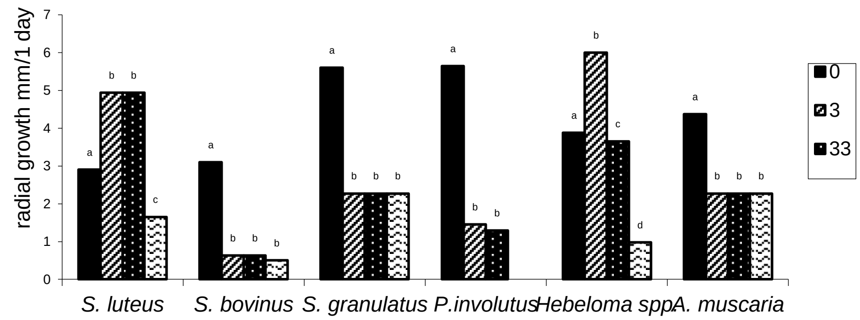

Figure 4. The growth rate (mm/1 day) of mycelium of mycorrhizal fungi on MEA medium with the different concentrations of lead.

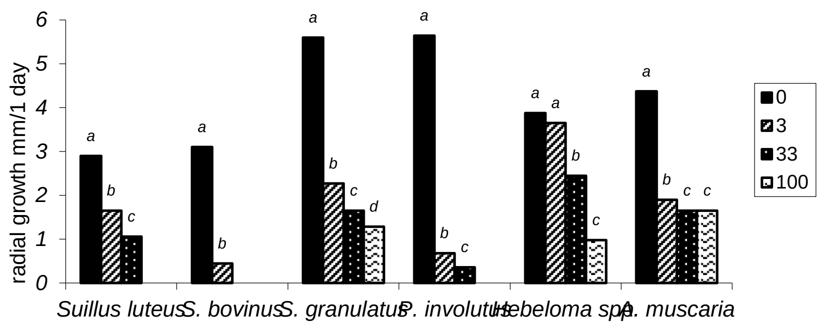

Figure 5. The rate of growth (mm day^{-1}) of mycelium of mycorrhizal fungi on MEA medium with the different concentrations of cadmium.

The results show that the average daily growth of all fungi was altered with the presence of heavy metals. The level of influence depended on the applied metal and concentration of that specific metal. The presence of zinc in all concentrations did not inhibit the growth of *Suillus luteus,* while its low concentrations showed stimulating effects to the growth of *Hebeloma* spp. The stimulating influence of copper and lead on the growth of *S. luteus* was reported, while this fungi exerted sensitivity to Cd. *Hebeloma* spp. showed good growth at higher concentrations of zink and lead, while its growth stopped at 100ppm of copper. *Amanita muscaria* and *Suillus granulates* were capable to grow in the presence of high concentrations of all metals.

4.1.2 The interaction between mycorrhizal fungi (the second pre-test)

The second pre-test was the assessment of interactions between the mycorrhizal fungi. The interactions between *Suillus granulatus, Suillus luteus, Suillus bovinus*, *Hebeloma* spp., *Paxillus involutus* and *Amanita muscaria* were examined in laboratory conditions by the "mixed cultures" method on MEA medium. Mycelia discs of 1cm in diameter were cut and placed at two ends of Petri plates. The assessment of the interaction between certain mycorrhizal species was determined as the ratio between their growth in the mixed culture and control (Mirić and Popović 2003). Five replicates were used per treatment. Results are presented in Table 6.

One of the most important characteristics of mixed inoculum is member's compatibility. Several mycorrhizal fungi can be used together in the production of seedling material. Their interactions are crucial for successful inoculation (Golubović Ćurguz et al. 2010b). Presence of antagonistic interactions in mixed inoculum can lower the effect of mycorrhization.

Presented results show that mixed cultures conditions caused growth inhibition of some fungi, while others responded by growth stimulation. *Paxillus involutus* caused stimulations of *Amanita muscaria* growth, while other fungi were inhibited. *Suillus luteus* stimulated the growth of *Paxillus involutus, S. granulatus, Hebeloma spp., Amanita muscaria. S. bovinus* stimulated the growth of *Hebeloma spp.*and *Amanita muscaria*. The presence of *S. granulatus* caused inhibition of all tested fungi. *Hebeloma spp.* positively influenced the mycelia growth of *Paxillus involutus, S.luteus, S. Bovinus* and *S. granulatus. Amanita muscaria* inhibited the growth of all cohabitants except *S. luteus.* The obtained results showed that *Suillus luteus* and *Hebeloma spp.* developed better with the presence of the most tested fungi.

Based on the results of the pre-tests, two fungi were chosen for further experimentation. *Other fungi stimulated Hebeloma spp. growth* and *Suillus granulatus* showed high heavy metals tolerance.

Table 6. The interaction between mycorrhizal fungi isolates (mm per 7 days).

Mycorrhizal fungi		***P. involutus***	***S. luteus***	***S. bovinus***	***S. granulatus***	***Hebeloma spp.***	***A. muscaria***
P. involutus	Alone	7.14					
	Mixed	7.14	2.34	2.70	2.58	2.28	8.77
	%		33	37	36	32	123
S. luteus	Alone	3.52					
	Mixed	3.86	3.52	3.17	3.92	4.34	4.30
	%	109		90	111	123	122
S. bovinus	Alone	6.21					
	Mixed	3.03	2.63	6.21	4.92	7.39	8.18
	%	49	42		79	119	132
S. granulatus	Alone	8.73					
	Mixed	7.00	5.37	6.80	8.73	3.58	4.75
	%	80	61	78		41	54
Hebeloma spp.	Alone	4.82					
	Mixed	17.29	8.92	8.39	8.00	4.82	3.15
	%	358	185	174	165		65
A. muscaria	Alone	5.35					
	Mixed	4.88	5.50	4.57	4.50	5.00	5.35
	%	91	103	85	84	93	

4.1.3 Performances of mycorrhizal Scots pine and Norway spruce seedlings in Majdanpek Mine deposal

Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L. Karst) mycorrhized and non-mycorrhized seedlings were obtained from the seeds sown:

- in peat with the mycorrhizal inoculum of *Hebeloma* spp. and *Suillus granulatus* (mycorrhized seedlings),
- in peat (non-mycorrhized seedlings, control).

Preparation of mycorrhizal inoculum was done according to the Marx et al. (1991) and Parladė et al. (2004). When Norway spruce and Scotch pine seedlings reached age two, they were re-planted into polyethylene bags filled with deposal-peat mixture. Deposal from location B was used as a substrate. Since this deposal is extremely unfavorable for plant growth, it was enriched with peat (1:1 ratio). The growth parameters and survival rates were monitored one more year. At the end of the vegetation period, the results were recorded (Tab. 7).

In our efforts to produce seedlings prepared to cope with challenges of Majdanpek Mine, we tried to imitate soil conditions by growing seedlings in deposol: peat mixture. Mycorrhized and non-mycorrhized seedlings (control) were grown in this substrate and compared at the end of the experiment. Scots pine mycorrhized seedlings were higher, with a thicker root collar diameter, longer root and higher biomass production, in comparison to control. Norway spruce mycorrhized seedlings had higher biomass production and longer roots. One of the most important parameter of successful reforestation is survival rate. In our study, mycorrhized seedlings showed much better results compared to non-mycorrhized. Influence of mycorrhiza on seedlings chances for survival was especially noticeable in the case of Norway spruce. The improved morphometric parameters and survival rates are consequences of well-formed, expanded root system, and all already mentioned benefits that those fungi enter into this symbiosis. Obtained results suggest that mycorrhized seedlings are better prepared for hush conditions of final placement.

Table 7. Plant growth parameters increment of mycorrhizal seedlings compared to control (%).

Parameter	Scots pine	Norway spruce
Shoot length	22	NI
Root collar diameter	10	4
Biomass	30	30
Root length	20	18
Degree of survival	15	45

(NI): no increase

4.2 Kolubara Mining Basin

The production of mycorrhizal seedlings isn't implemented in nursery practice of our country. For this situation, a good solution may be inoculation with PGPR. The advantage of inoculation with PGPR is the fact that it can be applied in nurseries, during seedling´s production, but also at the final placement. Inoculation of seeds and seedlings results in growth promotion. Inoculation can also be repeated several times during one growing season. Spaepen et al. (2009) consider inoculation performed at nurseries as a good technique, which induces vigorous growth and predispose better seedling performance after replanting in the field.

The substrates used in our experiments were deposals from the coalmine Kolubara, which covers huge areas scheduled for re-cultivation. The main defect of this substrate is nutrient deficiency but, on the other hand, such substrates are more suitable for exhibiting the full potential of PGPR (Egamberdiyeva 2007). The deposal was used in its original composition, without any amendments.

4.2.1 Performances of Scots pine and Norway spruce seedlings inoculated with PGPR (Gujaničić et al. 2012)

During the dormancy period, one-year-old, container-grown Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L. Karst) seedlings were re-planted into polyethylene bags filled with deposal from the coal mine Kolubara. The experiment was performed under nursery conditions (Nursery of The Faculty of Forestry, Belgrade, Serbia). At the beginning of the growing season, half of the seedlings were inoculated with 100ml of 10^8 CFU ml^{-1} of inoculum consisted of several PGPR strains (*Azotobacter chroococcum, Bacillus megaterium, B. circulans, B. licheniformis, B. pumilus, B. amyloliquefaciens*). The other half was left un-inoculated and got 100ml of distillated water. The results were collected at the end of the season, after nine months of the experimental period. Table 8 present the incensement obtained in comparison to non-inoculated control.

Table 8. The Plant growth parameters incensement after inoculation compared to control (%).

Parameter	Scots pine	Norway spruce
Shoot length	3	10
Root collar diameter	10	5
Biomass	43	34
Root length	23	63
Degree of survival	5	3

All observed growth parameters (shoot length, root collar diameter, biomass and root length) were improved in the presence of beneficial bacteria. Their positive influence was particularly apparent in cases of biomass production and root length. Compared to Scots pine, Norway spruce seedlings gave better results in terms of shoots and root length and biomass production. The survival rate was pretty high in both cases, inoculated and un-inoculated seedlings (92-97%).

Mixed inoculums confirmed to have a beneficial influence on seedlings grown in poor substrate. Inoculation improved biomass production is one of the most desired results in forestry. Root development is crucial for seedlings acclimatization on new environmental conditions.

4.2.2 Performances of Scots pine and black locust seedlings inoculated with PGPR (Karličić et al. 2015)

One-year-old, container-grown Scots pine (*Pinus sylvestris* L.) and bare root black locust (*Robinia pseudoacacia* L.) were re-planted into polyethylene bags filled with Kolubara deposal. The experiment was performed under nursery conditions (Nursery of The Faculty of Agriculture, Belgrade, Serbia). At the beginning of the growing season, half of the seedlings were inoculated with inoculum consisted of the following PGPR strains: *Bacillus licheniformis, Aeromonas hydrophila, Pseudomonas putida and Burkholderia cepacia.* Each plant got 100 ml of 10^8 CFU ml^{-1}. The other half got 100ml of distillated water. The data collected at the end of the growing season showed an increase in comparison to un-inoculated seedlings grown in the same substrate (Tab. 9).

The obtained results show increment of biomass production of both species. Also, measurements of root collar diameter confirm a stimulatory effect of applied PGPR. The survival rate was high for both treatments (91-95%).

The results suggest that PGPR inoculum used in the first experiment trigger a better response in Scots pine seedlings. This result emphasizes the fact that a series of field experiments need to be conducted in order to find the optimal inoculation formula for particular plant type and particular field conditions. PGPR influence highly depends on plant species, cultivar type, and genotype (Mehta et al. 2015).

Presented results suggest that selected PGPR have potential to induce faster growth of tested species and help in the recuperation of degraded ecosystems and establishment of their mature stage.

Table 9. Plant growth parameters incensement after inoculation compared to control (%).

Parameter	Scots pine	Black locust
Shoot length	NI	16
Root collar diameter	12	13
Biomass	22	19
Degree of survival	NI	4

(NI): no increase

5 Conclusions

Biological recultivation of post mining areas is burdened with variable chemical and physical properties of substrates. Comparison of substrates from Majdanpek and Kolubara Mine reveals substantial differences. Deposal from Majdanpek is extremely hostile, with low pH value, high concentrations of heavy metals, and lack of spontaneous plant invasion. On the other hand, Kolubara's deposal main shortage is lack of organic matter and that gives an opening for numerous pioneer species. The presence of these species enhances microbial activity and recovery processes in substrate, making the re-cultivation project much simpler compared to Majdanpek Mine.

In our efforts to get seedlings that are capable to cope with Majdanpek Mine challenges, we produced two types of seedlings; mycorrhized and non-mycorrhized seeds. Obtained two-year-old seedlings were re-planted into substrate, which unusual in nursery practice, consisted of deposal (location B) and peat mixture. The introduction of such substrate in seedling production was conducted with an aim to get ''targeted seedlings", seedlings adjusted and prepared for conditions that dominate at the reforestration area. Results obtained after one year showed that mycorrhized seedlings have better morphologic characteristics (shoot length, root length, root collar diameter, biomass) and expressed a higher survival rate.

Production of seedlings for Kolubara Mine reforestation was slightly different. We used seedlings produced under standard forest nursery practice and re-planted them into deposal. Thereafter, inoculation with mixed inoculums of PGPB were conducted and seedlings were observed for one more vegetative period. Results obtained at the end of the experiments showed that presence of PGPB improved morphologic characteristics of seedlings (shoot length, root length, root collar diameter, biomass). Both, inoculated and un-inoculated seedlings expressed a high level of survival. In the case of Scots pine, inoculum consisted of *Azotobacter chroococcum,* *Bacillus megaterium, B. circulans, B. licheniformis, B. pumilus, B. amyloliquefaciens* was more effective than the other one. This result emphasizes the fact that optimal inoculum formula demands comprehensive studies.

Results of all experiments suggest that inoculation with beneficial fungi and bacteria is a justified method for getting seedlings prepared to handle various, unfavorable issues of final plantation.

So far, the prevailing attitude was: High quality seedling material guarantee reforestation success. Very often high quality seedling material failed to meet expectations on areas with extreme conditions. Such experiences bring into focus high quality ''targeted seedling production". This approach suggests that imitation of final habitat conditions need to be introduced into the seedlings production. The easiest way to do that is by replacing standard nursery substrates with those that have the same/similar characteristics as substrates that cover areas planned for reforestation.

The real battle for the seedlings starts after being transplanted into the environment. Several factors affect the ability to adapt to new conditions, but vigorous and rapid growth of the root is essential at this point of reforestation (Chanway et al. 1991). Inadequate root performance is one of the main reasons why seedlings fail to accommodate to rarely optimal field conditions (Davis and Jacobs, 2005). Beneficial microorganisms and their ability to provoke better growth of roots can prevent this. Presented results confirmed the positive affect of fungi and bacteria on root development.

High quality seedling materials demands a satisfying nutrient supply. In this study, we tried to emphasize the already well known fact that beneficial microorganisms may be the suitable alternative for expensive, environmental damaging mineral fertilizers. Inoculation with mycorrhizal fungi and PGPR in nurseries is considered an easy to apply, low-cost technique.

In our experiments, mycorrhizal fungi and PGPB showed the ability to enhance plant growth and biomass production, which are the direct consequences of better nutrition. The presented study confirmed that microorganisms as "solutions from the nature" have potential to alleviate challenges that are an unavoidable component of every reforestation project.

6 Acknowledgments

This research was supported by the Ministry of Education and Science of the Republic of Serbia, Grant No. TR 31080 and FP-7 project AREA (316004).

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