IMPACT OF SEED EXPOSURE TO PLANT MATERIAL ON PLANT GROWTH AND DEVELOPMENT ON REMEDIATED ARID LANDS[1]

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Abstract. Remediation of land following surface mining requires the re-establishment of critical soil, plant, and microbial interactions on which the long-term sustainability of the site hinges. Current surface mine remediation practices may utilize topsoil with or without shredded plant material to overlay spoil. We evaluated whether the presence of such plant material may affect initial plant fitness and thus plant establishment. Tests were carried out in a greenhouse under controlled, replicated conditions common during early stages of remediation. Responses to seed exposure to plant material were species dependent. Plant growth parameters were linked to properties associated with the seed hull and seed surface and to functions associated with shredded plant material added to topsoil. Confirming the nature of these properties, hypothesized as microbial in origin, will be important for understanding factors critical to reclamation and management of disturbed sites, where native biological functions have been suppressed or fragmented. In degraded arid environments, such functions may govern micro-scale interactions that influence macro-scale processes.

Additional Keywords: land remediation, surface mine, plant fitness, non-specific inoculation

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Introduction

Soil fertility, potential for phytotoxicity, and water availability are the conventional variables considered when assessing and implementing land remediation protocols. For sustainable plant establishment and development, however, native or similar microbial populations must be reintroduced to the site. These microbial populations form relationships with plants and play a critical role in soil biological functions. Reintroducing native microbial populations is expected to reestablish soil and plant associated biological functions.

In the remediation of surface mines, a commonly used management practice includes overlaying post-mining surfaces either with topsoil that was collected prior to the mining disturbance and stored in stockpiles for extended periods, or by using topsoil freshly removed from nearby areas. In both cases, the re-established topsoil is expected to contain residues of the plant material present on and in the soil at the time of removal. In arid regions, however, during storage, there is minimal plant colonization and, commonly, no residual plant tissues can be identified after such storage. Alternatively, employing freshly removed topsoil (e.g. via direct haul) will include shredded plant remnants. In addition, the latter amendment is expected to be naturally colonized by endophytic, epiphytic, and rhizospheric microbial consortia, and thus is expected to reintroduce plant associative microbial populations to soils under remediation management.

These associative microbial populations may play a critical role in the improvement of plant fitness and stand establishment, as well as on the overall quality and success of surface mine re-vegetation efforts in arid environments. Thus, the primary goal of the experimental work presented here was to identify any impacts of such an amendment on the remediation potential of surface mine revegetation efforts.

Fungal and bacterial endophytes form systemic, *in planta*, relationships that are believed to be common for all plant species. Endophytes have been shown to systemically colonize plants intercellularly (Clay and Shardl, 2002; Schardl et al., 2004; Schulz and Boyle, 2005; Schulz and Boyle, 2006; Cheplick and Faeth, 2009). Microscopy, culturing of isolates and genetic sequencing techniques have identified and confirmed endophytes to exist in plant roots, shoots, leaves, and seed (Kremer, 1987; Barrow and Aaltonen, 2001; Barrow, 2003; Barrow et al., 2004; Lucero et al., 2006; Lucero et al., 2008ab; Lucero et al., 2011).

Microbial endophytes contained in living plants interact and colonize the rhizosphere and surrounding soil. Microbial endophytes also enter the soil environment via deposition of plant

material (Omancini et al., 2004; Van Hecke et al., 2005; Franzluebbers and Hill, 2005; Franzluebbers, 2006). The degree to which any such endophyte successfully colonizes the rhizosphere and completes horizontal transmission processes is likely dependent on abiotic environmental conditions, plant species, and endophyte type. Soil microbial communities have been shown to form endophytic relationships with deposited seed and rhizospheric microbial communities have been identified in plant tissues, indicating horizontal transfer and thus recruitment of endophytes via soil (Gallery et al., 2007; Kluger et al., 2008; Khidir et al., 2010; Knapp, 2012). However, the mechanisms of selection by plants have not been widely characterized. Certain microbial communities in arid soil environments, especially the group informally known as the dark septate fungi (DSF), have been found to inextricably link neighboring plants and their endophytic populations (Barrow and Osuna, 2002; Lucero et al., 2006; Khidir et al., 2010; Porras Alfaro et al., 2011; Knapp et al., 2012) and also link plants with biotic soil crusts and rhizosphere soils in a fungal loop, akin to mycelia mycorrhizal networks (States and Christensen, 2001; States et al., 2003; Porras-Alfaro et al., 2007; Collins et al., 2008; Green et al., 2008).

Vertical transmission of endophytes through seed progeny has been well-documented (Kremer, 1987; Schardl et al., 2004; Lucero et al., 2008a; Gallery et al., 2007; Lucero et al., 2011; Johnston-Monje and Raizada, 2012). On the other hand, horizontal transfer may be the most common path for endophyte transfer from plant to the environment and among plants (Saikkonen et al., 2004; Gallery et al., 2007; Kluger et al., 2008). Co-cultivation or inoculation experiments have confirmed horizontal transfer of endophytes between plant species (Lucero et al., 2008a) and have resulted in altered plant phenotypes that enhanced plant fitness under abiotic stress suggesting transfer of function associated with horizontal transfer of endophytes (Lucero et al., 2008a). However, it is thought that both horizontal and vertical transmission occurs within a single host (Afkhami and Rudgers, 2008).

Evidence of enhanced plant fitness due to endophyte colonization has been shown. Exclusion of endophytes associated with enhanced thermo tolerance resulted in diminished plant growth (Redman, 2002). In arid environments, endophytes, particularly DSF, enhance host heat and drought stress. Endophytic mechanisms of enhancing plant thermo tolerance and drought stress include melanized cell walls and endophytic adjustment of plant osmotic potential (Bell and Wheeler, 1986; Ravel et al., 1997; Elemi and West, 1995; Elemi et al., 2000; Redman et al., 2002;

Pennisi, 2003; Knapp et al., 2012). DSF that exist cryptically in some arid ecosystem plant species, and are associated with arid environment biotic crusts and rhizosphere soils, have been shown to facilitate the transformation and transportation of C, N, and P between plants and soils via hyphal networks (States and Christensen, 2001; Barrow and Osuna, 2002; States et al., 2003; Porras-Alfaro et al., 2007; Collins et al., 2008; Green et al., 2008).

Root endophytic fungi have also been shown to enhance P uptake by plants likely through alteration of host biochemistry as plant roots colonized with endophytic fungi exuded exponentially more Fe^{3+} reductase and had significantly larger phenolic concentrations in shoots and roots (Malinowski et al., 1998, 1999 and 2000). Alteration of other host chemical processes through *in planta* production of N and P assimilating enzymes has also been reported (Gasoinbi et al., 1997; Maccheroni et al., 1998; Sherameti et al., 2005).

It is evident that plant-microbial interactions enhance plants' ability to cope with abiotic and biotic stresses resulting in greater plant fitness. Presence of plant residues in the soils to be remediated is expected to favor the establishment of such interactions and thus result in enhanced plant establishment. For this reason, it is important to identify the role of the addition of such plant material to soils on plant growth and development within the framework of arid region surface-mine revegetation and framed the objectives of this study:

1) Identify germination rates between plant species and the significance of the pre-planting seed surface disinfection.
2) Elucidate the impact of seed treatments and seed exposure to plant material on plant growth and development in a replicated controlled environment.

Methodology

Overview

A series of experiments were designed to identify the effect of seed surface disinfection, expected to eliminate any seed epiphytes, on seed germination, plant growth and development. Thus, firstly a germination efficiency test was carried out to examine if the inclusion or exclusion of seed associated epiphytes would alter the germination rate of selected mine site reclamation species. Next, a greenhouse (GH) experiment was designed to examine of the impact of germinant exposure to small amounts of ground plant material – possibly serving as a nonspecific microbial/endophytic inoculum but offering no significant nutrient source — on plant growth and development under a scenario simulating field conditions of coal site reclamation near Fruitland,

NM. Treatment impacts were estimated through measuring plant growth parameters. Evidence for putative endophyte transfer and impact on plant biometric parameters offer an insight into the role of the presence of plant material in the reclamation process particularly in arid environments. Sequence based phylogenetic inventory of inoculum confirmed the presence of endophytic, epiphytic, or rhizosphere microbial consortia in the inoculum. Future phylogenetic analysis of collected plant tissue, soil, and spoil will allow for a more detailed examination of the dynamics of microbial transfer and plant recruitment of microbial consortia between soil, host plant, and any donor plant.

Germination Efficiency Test

Germination is governed by a number of factors including species, seed age, moisture, temperature, light or darkness (Bonner, 2008), and may be enhanced by pre-treatment such as scarification or cold stratification (Bonner, 2008). Considering the time, effort and resources put into seed broadcasting and irrigating large swaths of land, as well as the cost of seed itself, identification of germination rates between species and batches of seed is critical when estimating the success of a reclamation operation. Tested seeds were representative of species commonly used for surface coalmine remediation in North-Western New Mexico: *Krascheninnikovia lanata, Bouteloua gracilis, Sarcobatus vermiculatus, Pleuraphis jamesii, Sphaeralcea munroana, Sporobolus airoides, Achnatherum hymenoides, Atriplex canescens,* and *Atriplex confertifolia*. Seed germination testing was carried out at the New Mexico Department of Agriculture (NMDA) Seed Lab following Association of Official Seed Analysts (AOSA) and US Department of Agriculture (USDA) protocols (AOSA, 2009; USDA 2008) (Table 1).

Two germination treatments were considered: 1) a positive control wherein seed was only chemically or mechanically treated as advised by AOSA and USDA protocols and 2) seed was preliminarily dehulled to the naked caryopsis followed by disinfestation with Zerotol™[3] (surface disinfested seeds) (Lucero et al., 2008a) before germination testing. Seed disinfestation was achieved by soaking the de-hulled seed for 30 minutes in a 1:100 Zerotol™: sterilized water

[3] Reference herein to any specific commercial products, does not constitute or imply its endorsement, recommendation, or favoring by New Mexico State University.

solution followed by a rinse with sterilized water. A total of 800 seeds per species (400 non-treated, 400 de-hulled-disinfested) were used to determine germination percent.

Table 1. Seed Germination Parameters

Species	Temp (˚C)	Substrata	Light	Specific Notes/Requirements
A. hymenoides	15	P	No	Pre-chill at 5˚C for 4 weeks and test for 21 additional days; treat 15g seed in 200mL of 67% H_2SO_4 for 40 minutes
A. canescens	15	B	No	Dewing seed; 4-24 weeks cold stratification
A. confertifolia	15	T	No	Mechanical treatment to remove or fissure seed coat; Pre-chill at 1 ˚C then at5/15 ˚C for 12 weeks
K. lanata	15	T	No	Imbibe diaspores; Pre-chill at 0 to 5˚C for 4-5 days
S. vermiculatus	15	B	Yes	Rub to remove membranaceous wings and empty seed
S. munroana	15-30	B	Yes	Scarify seed before planting on blotter
S. airoides	20-30	B	Yes	Pre-chill for 14 days; soaked in 0.02% KNO_3
P. jamesii	20-30	B	No	None
B. gracilis	20-30	P	Yes	Rinse with D/I water; soaked in 0.02% KNO_3

B=Between blotters **T**=Paper toweling **P**=covered petri dishes with: two layers of blotters or three thickness of filter paper (USDA, 2008; AOSA, 2009).

Greenhouse Test

Plant species (Table 1) were grown in pots of soil and spoil (overburden) collected from the reclamation site and layered (soil overlying spoil) in the pots to simulate the site conditions. To assess the possible role of seed endophytic or epiphytic microbial consortia, surface disinfested or fresh seeds were used. The possible role of the naturally occurring microbial consortia was verified by employing a mixture of ground plant tissues collected from species native to the remediation site.

Treatments For each species, 3 treatments were considered. Each treatment was carried out in triplicate. The first treatment (Trmt A) used fresh untreated seeds. A second treatment (Trmt B) used dehulled and surface disinfested seeds (as described under Germination Efficiency). The third treatment (Trmt C) employed dehulled and surface disinfested seeds, and 1g native plant material mix added to the soil pots. Dehulling and disinfestation of seed serves as a method of exclusion of seed-borne epiphytes and hull endophytes; however, inherently, cryptic seed-borne endophytes may exist within the seed despite treatment.

Native plant material mix (MIX) The native plant material mix, which served as a potential source for plant associated microbial consortia, consisted of a mixture of above-ground tissues of *S. airoides*, *S. vermiculatus*, *A. hymenoides*, *S. munroana*, *A. canescens* and *A. confertifolia*. Plants were collected from undisturbed reference sites at BHP Navajo Coal Company's Navajo Mine surface coalmine near Fruitland, NM. Plant material was ground in a coffee grinder to a coarse, mulch-like consistency. Between species, the coffee grinder was cleaned, surface-sterilized with 70% ethanol solution, and air dried. MIX was prepared by mixing 7g of ground plant material of each plant species. Mixing was carried out in a sterile container and shaken manually to achieve homogeneity. A sub-sample of the inoculum was retained for identification for fungal and bacterial diversity.

Microbial composition of MIX, plant material, soil and spoil DNA was extracted using MoBio PowerPlant® DNA or MoBio PowerSoil® DNA extraction kits. For plant material, soil and spoil, DNA was extracted from three 0.25g aliquots. One composite sample of the three extracted samples was used for analysis. The extracted sample was analyzed for fungal and bacterial populations using high-throughput tag encoded 454 pyrosequencing as described in Lucero et al., 2011. Metagenomic analyses, including sequence quality control, hierarchical taxa assignment, clustering and MEGA Blast sequence alignments were carried out using the pipeline available at (www.camera.calit2.net, Sun et al., 2011), MEGAN4 (MEtaGenome ANalyzer, http://ab.inf.uni-tuebingen.de/software/megan , Huson et al., 2011) and RDP (Ribosomal Database Project http://rdp.cme.msu.edu/classifier/classifier.jsp , Wang et al., 2007). Similarity comparisons of microbial taxa associated with individual samples were visualized in VENNY (http://bioinfogp.cnb.csic.es/tools/venny/ , Oliveros, 2007).

Soil and spoil collection Spoil and soil were obtained from the Navajo Mine 16 miles SW of Fruitland, San Juan County, NM 87416 (for their chemical properties see Table 2). The Navajo Mine covers approximately 133.55 km^2. The mine area is located on the west flank of the San Juan Basin. Topsoil was collected from stockpiles where the material had been stored for several years. The major sources of suitable topdressing collected from the site are a complex of sandy, coarse-loamy and fine-loamy textured soils derived from eolian and alluvial sources. Overburden is a geologic formation composed of sandstone, siltstone, claystone, mudstone and shale. Textures of crushed samples are most frequently clay loams and silty clay loams. The Navajo Mine Permit Application provides detailed surveys of mine site geology and soil and overburden characteristics.(http://www.wrcc.osmre.gov/Current_Initiatives/Navajo_Mine/Permit.shtm) Texture

analysis of collected soil and spoil used in the GH experiment found the soil to be a sandy loam and the spoil to be a sandy clay loam. There was no evidence of plant growth on the soil at the time of sampling and no evidence of residual plant material in the soil or spoil. Soil and spoil was sieved through a 9.5 mm sieve on site prior to transport.

Table 2. Stock soil and spoil chemical properties

	Metal concentrations (mg kg^{-1})							
	K	Mg	Ca	Na	Zn	Mn	Fe	Cu
SOIL	78	400	2941	303	0.2	1	1	0.2
SPOIL	269	407	510	1963	3.5	11	6	2.8

	Standard fertility parameters (mg kg^{-1})								
	OM %	ENR* (kg ha^{-1})	pH	CEC	TKN %	NO_3-N (mg L^{-1})	Soluble Salts (mmhos cm^{-1})	P (mg kg^{-1}) Weak Bray	P (mg kg^{-1}) Strong Bray
SOIL	1.5	80.6	8.5	19.5	0.14	4	1.3	4	18
SPOIL	2.5	50.4	7.5	38	0.23	6	2.4	10	81

	Percent Base Saturation			
	K	Mg	Ca	Na
SOIL	1	16.9	75.3	6.8
SPOIL	1.8	8.8	67	22.4

*ENR: estimated nitrogen release.

Growth conditions and initial planting PVC pipes 46 cm long, and 10 cm in diameter were used as plant containers. Commercial weed barrier was secured at the bottom of each container to allow drainage without loss of substrate. A 13 cm of layer of spoil at the bottom of the containers was overlaid with 23-28 cm of topsoil. This layering follows the general practices used in surface mine remediation. Soil moisture was maintained at field capacity.

Fifteen seeds of one species were planted in each container. After germination, one plant was retained per container a total of 81 plants (9 of each species). Three non-seeded control pots were also prepared. Containers were then placed into one completely randomized block. Pots were watered as needed with tap water to maintain field water capacity moisture conditions. The experiment was carried out in a greenhouse between March 2011 and November 2011 under controlled temperature (18-37 ˚C) and light cycling conditions (16 hrs light and 8 hrs dark daily).

Initial planting occurred on 23 March 2011. Containers wherein no seed germinated two months after initial planting were reseeded according to treatment type. Containers without germinants following the first reseeding were subjected to no more than three reseedings. The final reseeding occurred on 9 June 2011. Destructive harvest began 15 August 2011 and continued through November 2011.

Growth monitoring and sampling Dates of germination were recorded for each individual plant. Plant height was recorded for each plant three times weekly, allowing the development of growth curves for individual plants, species, and species within treatment types.

To accurately separate sample layers and to avoid disturbing root structure, containers were placed in a vice and carefully cut vertically on two sides with a reciprocating saw. From each container spoil, soil, soil crust, and a mix layer (a 4 cm layer at the interface between soil and spoil) were separated and placed into sterile bags. This approach allowed for documentation of an undisturbed plant-soil system, which made it possible to accurately separate soil from spoil samples as well as to distinguish soil-rhizosphere and spoil-rhizosphere samples. All sampling equipment was surface sterilized with 70% ethanol and air dried between samples. Following sampling, roots were rinsed, air dried, and weighed; extended root length was measured, and root volume was measured by liquid displacement (Harrington et al., 1994; Pang et al., 2011). Plants were separated from roots by severing the stem at 1-2 mm above the soil surface; above ground plant height and weight were recorded.

Soil and spoil chemical analysis Unless otherwise mentioned all soil and spoil samples were analyzed following procedures as in the indicated sections of Methods of Soil Analysis (Page, 1982). Soil and spoil samples were homogenized before sampling for analysis. Percent organic matter (OM) was evaluated using the Walkley Black method (Walkley and Black, 1934). Saturated paste extract pH values were obtained using a bench-top glass electrode-pH meter method (section 12-2.65). Pre-test soil and spoil (section 8-3), were tested for their cation exchange capacity (CEC, section 24-2.3) total P and K values (section 13-2.2), salinity by the 1:2 soluble salt extract method (section 10-2.3.2), Fe, Zn, Mn, Cu by Diethylenetriaminepentaacetic acid (DTPA) extract (sections 17-4.3 and 19-3.3), and total sulfur (section 28-2.2.1). NO_3–N and Total Kjeldahl Nitrogen (TKN) were tested according to the procedures listed in Western States Laboratory Soil and Plant Analytical Methods (Gavlak et al., 2003). NO_3 was measured using an ion-selective electrode (ISE) meter with $AlSO_4^{-2}$ extract (section S-3.20); TKN was measured by automated combustion methods using a LECO Fp-428 N analyzer (section P-2.20).

Statistical Analysis Statistical analyses were carried out separately for each plant species using Minitab® 16.1.1 (Minitab Inc., 2010). This included analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) for a confidence interval of 90% (CI_{90}). A large α (i.e. 10%)

was applied due to low sample size (n=3) (Antunes et al., 2006). This allowed estimates of statistical trends along the considered treatments.

Results

Germination Efficiency

Germination rates varied significantly between species (Tables 3 and 4) and statistically significant rate differences were observed between treatments. Dehulling and disinfestation (seed treatment) led to decreases in germination rates for *K. lanata, B. gracilis* (p=0.04) *and A. canescens*. Increases in germination correlated to seed treatment were observed for *S. vermiculatus* and *P. jamesii* (p=0.09). No differences in germination associated with the seed treatment were observed for *S. airoides, S. munroana, A. hymenoides* and *A. confertifolia*. *B. gracilis, P. jamesii* and *S. airoides* had germination rates above 60% while all other species had germination rates below 40%.

Soil and Spoil Chemical Parameters

Soil and spoil were sampled dry (water content < 0.01g g^{-1}). Stock soil and spoil chemical and fertility parameters (Table 2) indicate that both the soil and spoil were alkaline, high in soluble salts and Na, and contained free carbonates. Stock spoil was high in Cu. Macronutrient availability was low in both soil and spoil. Given the residual coal in the spoil the estimated organic matter (OM) percent was measured as higher in spoil than soil. Soil and spoil pH and OM values did not differ by species and treatment but plants had a measurable impact. In the presence of plants soil pH decreased by 0.4-0.7 and spoil pH increased by 0.3-0.6; similarly OM for both soil and spoil increased by 1.75% ± 0.25% and 2.8% ± 0.3%, respectively. Control soil (watered only) retained stock soil pH (8.5 ± 0.2) and OM (1.5 ± 0.2) values. Control spoil (watered only) had higher pH values (8.5 ± 0.1) than stock spoil, and higher OM values (2.5 ± 0.3) than stock spoil.

Table 3. Significance (P-values) of treatment effects for plant biometric parameters.

	S. airoides	*B. gracilis*	*P. jamesii*
Germination Rate (%)	0.19*	**0.04**	**0.09**
Root Mass (g)	**0.06**	0.64	0.20
Root Volume (mL)	**0.09**	0.61	0.13
Root Length (cm)	0.70	0.22	0.40
Shoot Mass (g)	**0.02**	0.88	0.13
Plant Height (cm)	0.36	**0.07**	0.15
Plant Biomass (g)	**0.03**	0.65	0.16
Shoot Mass : Plant Height (g cm^{-1})	**<0.001**	0.56	**0.10**
Shoot Mass : Root Mass (g g^{-1})	0.96	0.93	0.31

*P-values generated by ANOVA ran at a 90% confidence levels

Plant Growth Parameters

Treatment effects on plant growth parameters were evident but varied by species. *S. airoides*, *B. gracilis*, and *P. jamesii* were the only species to consistently germinate across treatments and replications to justify subsequent data collection and analysis. All *S. airoides*, *B. gracilis*, and *P. jamesii* were planted between March 23rd and April 4th, and harvested between October 14th and November 8th. All germinated within five days of planting, and did not require reseeding. During the simulated growth season some *B. gracilis*, *S. airoides*, and *P. jamesii* were also seeded. In general, with exposure to MIX plant growth parameters were enhanced for *S. airoides*, decreased for *P. jamesii*, and without any statistical effect on *B. gracilis*. It is important to note variations in plant vigor associated with our seed source for plants in this experiment. Some plants in this experiment exhibited plant growth responses dissimilar to other plants of the same species within a single treatment. Such variation is common among native plants, which are typically exposed to highly variable selective pressures. Native plants are expected to contain high levels of genetic variation and more variable microbial associations, which interact to produce high plant-to-plant phenotypic variability. This variance compounded by a small sample size, may have inflated confidence intervals for particular growth parameters.

Root mass, length, volume Seed exposure to MIX was correlated to larger root mass (p=0.06) and volume (p=0.09) for *S. airoides*, but these were marginally negatively correlated for *P. jamesii* (p=0.13 and p=0.20 respectively); no treatment effect could be observed for *B. gracilis* (Table 3). All species and treatments, with the exception of Trmt C *P. jamesii*, had total root lengths over

45cm. *S. airoides* root length did not vary across treatments. The presence of spoil did not limit root development, nor were there differences in spoil colonization based on treatment. Visually, *S. airoides* was the most vigorous spoil colonizer across all treatments.

Shoot mass, plant height and shoot mass: plant height ratio Shoot mass was larger ($p=0.02$) for MIX exposed *S. airoides* in comparison to the untreated or dehulled-disinfested-only seed treatments. *B. gracilis* shoot mass was the same ($p=0.88$) across treatments. For *P. jamesii*, exposure to MIX was associated with marginally lower shoot mass ($p=0.13$). For *S. airoides*, no difference ($p=0.36$) in plant height was recorded across treatments, although MIX exposed plants were generally taller. For *B. gracilis*, treatments B and C resulted in similar plant height, both generally taller than plants grown from untreated seed. For *P. jamesii* no statistical difference in plant height due to treatment was identified, but exposed plants were generally observed to be shorter. *S. airoides* shoot biomass per plant height (g cm^{-1}) was greater ($p<0.001$) for both B and C treatments. For *B. gracilis* and *P. jamesii*, exposure to MIX was associated with shorter plants, but without significantly different shoot biomass per plant height ratios (Table 3 and Table 4).

Plant biomass Dehulled disinfested and MIX exposed *S. airoides* had larger ($p=0.03$) biomass (shoot mass + root mass g) than plants grown from untreated seed. No differences in biomass for *B. gracilis* or *P. jamesii* were observed across treatments (Table 3 and Table 4).

Shoot mass: root mass ratio For *S. airoides*, *B. gracilis*, and *P. jamesii* there were no differences in the shoot to root mass ratios across all treatments (Table 3 and Table 4).

Growth rate Growth rates, as described here, were obtained from plant height time-series (Fig.1). Exposure to MIX accelerated *S. airoides* growth. With or without exposure to plant material, an initial spike in growth rates among plants obtained from disinfested seeds was observed for all species, but overall growth was not significantly different than for the untreated seed plants, except in the case of *B. gracilis* (Fig. 1). With or without MIX exposure, seed disinfestation accelerated growth of *B. gracilis*.

Table 4. Impact of treatments on germination and growth biometric parameters.

	S. airoides			***B. gracilis***			***P. jamesii***		
	Germination test: Seed treatments								
Germination Rate (%)	Surface Disinfested		Untreated	Surface Disinfested		Untreated	Surface Disinfested		Untreated
	87.3 ± 0.5^{a}		89.0 ± 1.4^{a}	87.3 ± 4.1^{b}		92.0 ± 1.4^{a}	69.5 ± 0.9^{a}		64 ± 5.8^{b}
	Greenhouse test: Treatments								
	A	B	C	A	B	C	A	B	C
Root Mass (g)	24.9 ± 18.0^{b}	51.1 ± 13.3^{ab}	75.9 ± 25.4^{a}	37.7 ± 13.3^{a}	62.2 ± 55.4^{a}	36.5 ± 15.3^{a}	42.5 ± 36.3^{a}	32.8 ± 9.6^{a}	4.6 ± 6.2^{a}
Root Volume (mL)	23.3 ± 16.6^{b}	47.3 ± 15.7^{ab}	63.3 ± 18.5^{a}	30.0 ± 8.2^{a}	45.0 ± 28.4^{a}	32.3 ± 10.6^{a}	31.2 ± 18.7^{a}	27.0 ± 10.0^{a}	6.0 ± 7.4^{a}
Root Length (cm)	49.2 ± 10.3^{a}	53.5 ± 0.5^{a}	49.0 ± 4.4^{a}	45.1 ± 5.2^{a}	59.0 ± 10.7^{a}	55.7 ± 8.9^{a}	53.6 ± 8.1^{a}	54.7 ± 11.3^{a}	36.0 ± 11.0^{a}
Shoot Mass (g)	12.0 ± 4.4^{b}	28.6 ± 9.1^{ab}	41.3 ± 9.6^{a}	15.0 ± 6.2^{a}	18.2 ± 9.8^{a}	16.1 ± 4.8^{a}	11.5 ± 3.5^{a}	13.4 ± 6.3^{a}	3.8 ± 4.6^{a}
Plant Height (cm)	45.7 ± 4.2^{a}	41.4 ± 6.0^{a}	54.9 ± 18.0^{a}	39.3 ± 3.9^{b}	65.6 ± 15.2^{ab}	56.0 ± 9.0^{a}	39.1 ± 8.3^{a}	39.7 ± 2.6^{a}	26.7 ± 9.2^{a}
Plant Biomass (g)	36.9 ± 21.0^{b}	79.7 ± 22.1^{ab}	112.3 ± 33.4^{a}	52.8 ± 15.3^{a}	80.4 ± 62.3^{a}	52.6 ± 20.0^{a}	54.0 ± 39.8^{a}	46.2 ± 15.4^{a}	8.4 ± 10.8^{a}
Shoot Mass:Plant Height ($g\ cm^{-1}$)	0.3 ± 0.1^{b}	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}	0.4 ± 0.2^{a}	0.3 ± 0.1^{a}	0.3 ± 0.07^{a}	0.3 ± 0.03^{a}	0.3 ± 0.1^{a}	0.1 ± 0.1^{a}
Shoot Mass:Root Mass ($g\ g^{-1}$)	0.6 ± 0.3^{a}	0.6 ± 0.06^{a}	0.6 ± 01^{a}	0.4 ± 0.2^{a}	0.4 ± 0.2^{a}	0.5 ± 0.1^{a}	0.4 ± 0.3^{a}	0.4 ± 0.09^{a}	0.6 ± 0.9^{a}

Statistical analyses were carried out separately for each parameter and individual species. Data represents the Mean ± 10%. Superscripts indicate statistical differences according to Tukey's HSD for a family error rate of 10%; values followed by the same letter are not significantly different. Treatments are abbreviated as follows: A=Untreated seeds; B=Dehulled and surface disinfested seeds; C=Dehulled and surface disinfested seeds exposed to inoculum.

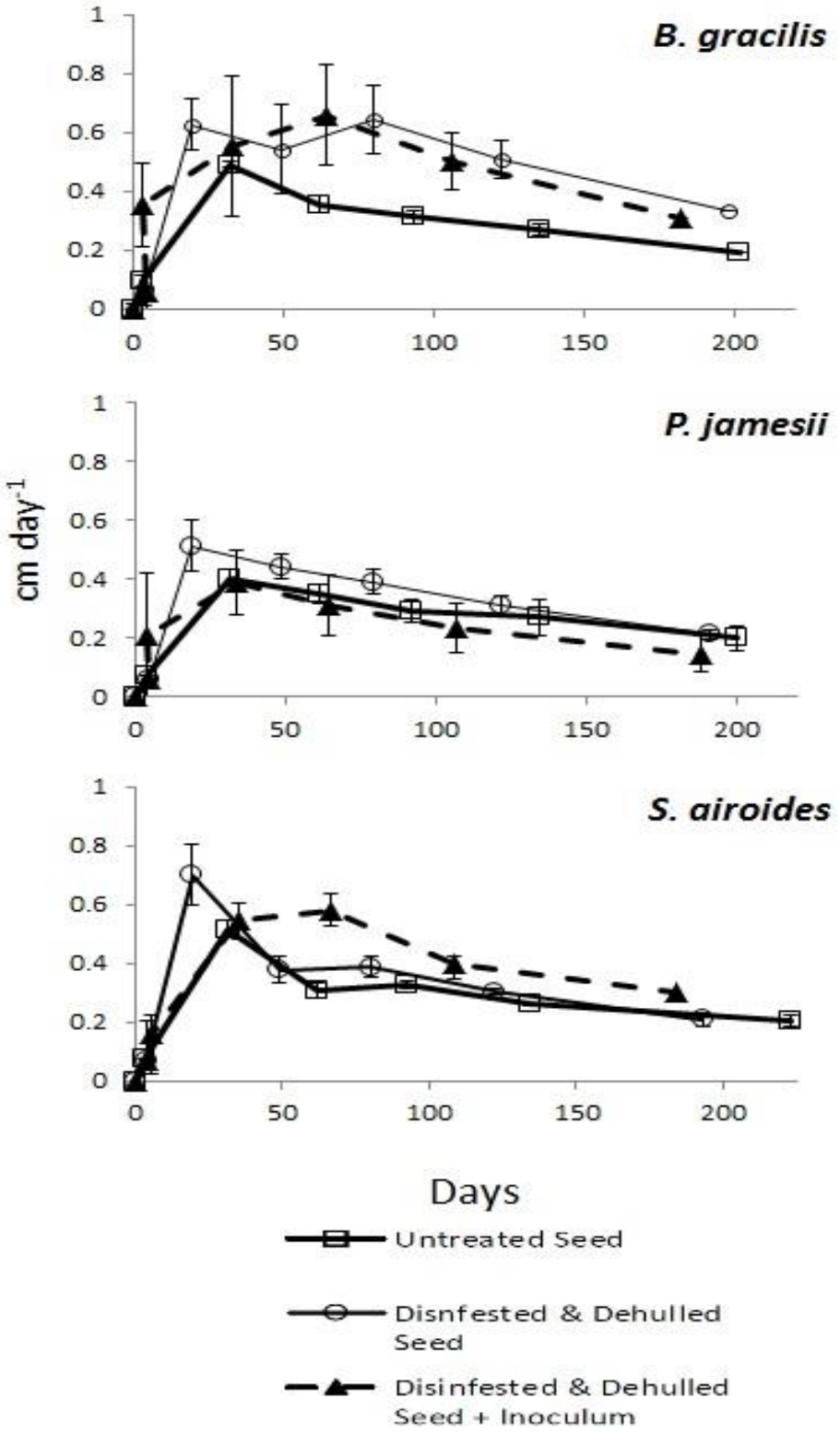

Figure 1. Time series growth rates; plant height (cm day^{-1}). Error bars represent 90% CI

Microbial composition of MIX, plant material, soil and spoil

Genetic sequencing and subsequent metagenomic analysis revealed a diverse microbial community contained in MIX (Fig. 2), plant material (Fig. 3), and fallow soil and spoil control treatments (Figs. 6, 8, 11, 13). MIX contained 17 bacterial orders and 3 fungal orders (Fig. 2). Microbial consortia contained in the ground plant material are representative of known endophytic, epiphytic, and rhizosphere microbial consortia (Porras-Alfaro et al., 2008; Rodriguez et al., 2008; Porras-Alfaro et al., 2011). Genus level taxonomic comparisons of microbial communities show an effect of seed treatment and germinant exposure to MIX on seed and plant microbial populations. Seed de-hulling and disinfestation removed bacterial (Fig. 4) and fungal (Fig. 9) consortia associated with the seed hull, leaving some seed-associated bacterial and fungal populations. Seed treatment resulted in a 93.9% reduction of bacterial genera associated with *B. gracilis* seed and a 53.6% and 91% reduction in bacterial genera for *S. airoides* and *P. jamesii* (Fig 4). For each plant species, a minority of bacterial taxa was unique to dehulled and disinfested seed, and another, smaller, minority was shared between untreated and treated seed (Fig. 4). For *B. gracilis*, seed treatment has shown no unique fungal genera in dehulled and disinfested seed; one fungal genus was shared between untreated seed and treated seed (Fig. 9). For *S. airoides*, treatment has shown four unique fungal taxa to be found in dehulled and disinfested seed; one fungal genus was shared between untreated and treated seed (Fig. 9). For *P. jamesii*, treatment resulted in four unique fungal taxa found in dehulled and disinfested seed; one fungal genus was shared between untreated seed and treated seed (Fig. 9).

Plants grown from dehulled and disinfested seed share microbial populations with dehulled disinfested seed unique of microbial populations associated with fallow control soil and spoil (Fig. 6 and Fig 11) and untreated seed and plants grown from untreated seed (Fig. 7 and Fig. 12). Alternatively, plants grown from untreated seed contain microbial populations found in untreated seed but not microbial populations found in dehulled disinfested seed and plants grown from dehulled disinfested seed (Fig. 7 and Fig. 12), or control soil and spoil (Fig. 6 and Fig 11). Germinants grown from dehulled and disinfested seed exposed to MIX share bacterial and fungal populations also found in MIX (Fig. 3) but not in control soil and spoil (Fig. 6. and Fig. 11), untreated seed (Fig. 5 and 10), dehulled and disinfested seed (Fig. 8 and Fig. 13), or germinants grown from untreated seed or germinants grown from dehulled and disinfested seed not exposed to MIX (Fig. 6 and Fig. 11).

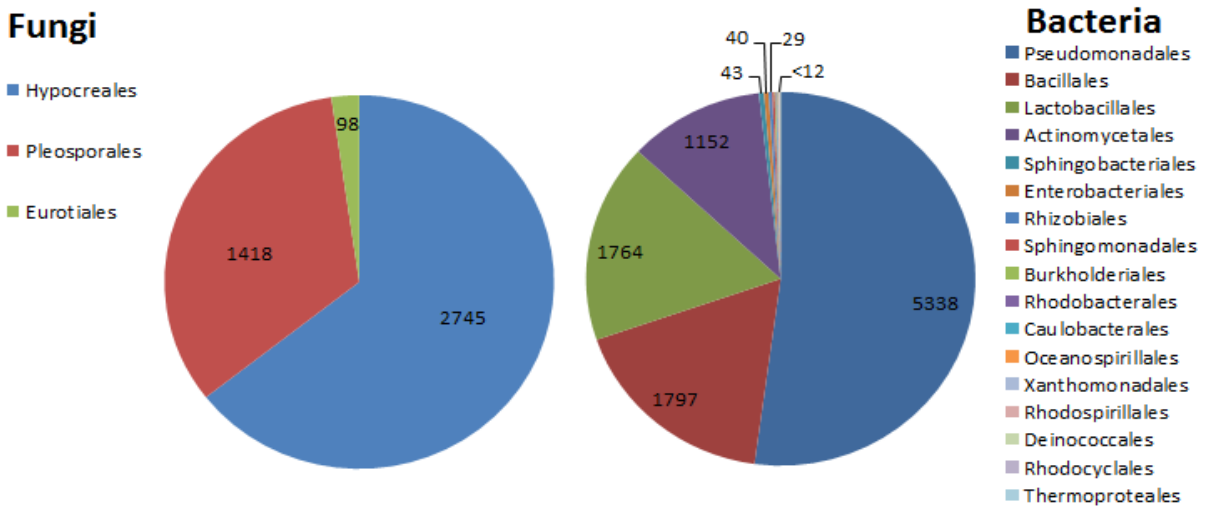

Figure 2. Taxonomic fungal and bacterial diversity of inoculum at Order level; Operational Taxonomic Units (OTUs) assigned to each order are listed as absolute counts. A total of 10,235 bacterial and 4,261fungal OTUs were identified.

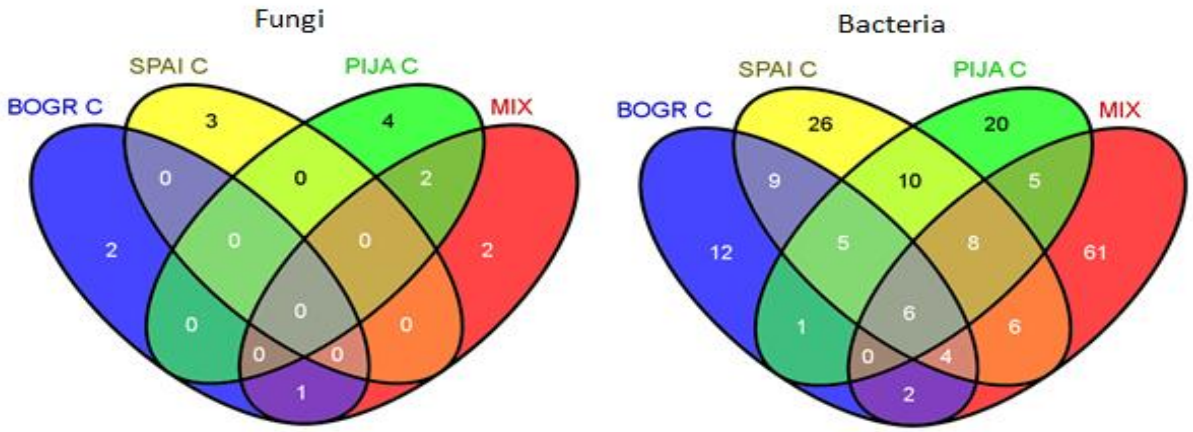

Figure 3. Genus level taxonomic similarity comparison of fungal and bacterial OTU's between the inoculum mix and the plants exposed to the same inoculum (Trmt C).

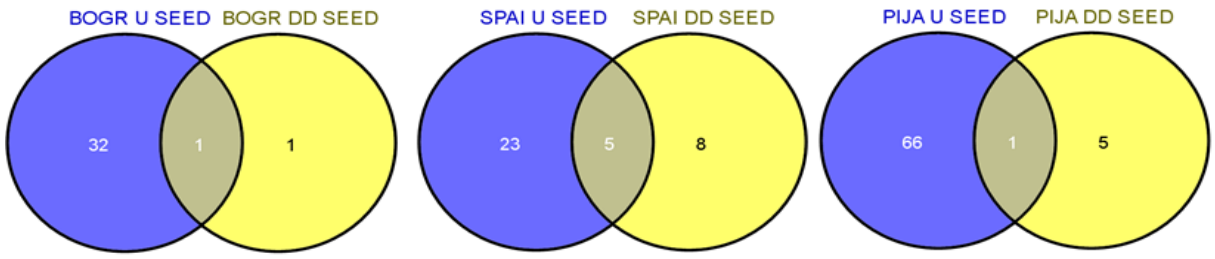

Figure 4. Evidence for seed and seed hull bacterial taxonomic putative specificity or similaritiy. Bacterial populations of untreated seeds (U) compared to dehulled and surface disinfested seeds (DD). Note that a minority of taxa were perceived unique to DD seeds, likely due to extraction and sequencing bias associated with low abundance taxa.

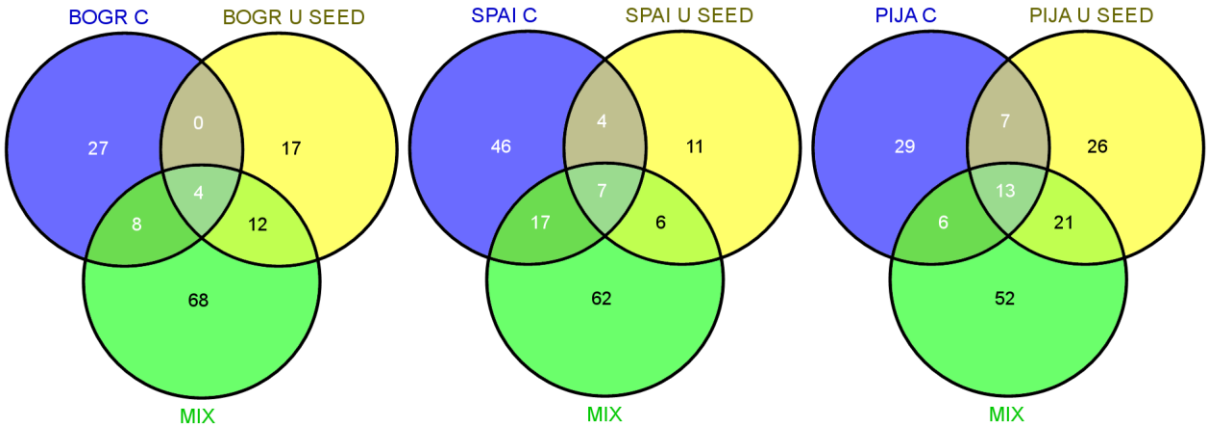

Figure 5. Genus level taxonomic similarity comparison between the bacterial populations in the untreated seed (U), plants grown from dehulled and and surface disinfested seed (DD) and exposed to plant material inoculum (Trmt C), and inoculum mix (MIX).

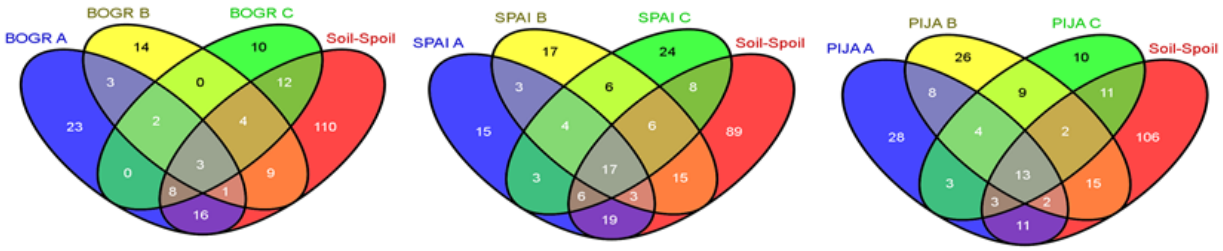

Figure 6. Genus level taxonomic similarity comparison between the bacterial populations in the fallow soil and spoil control treatments and the populations recovered from plants grown from untreated seeds (A), seeds dehulled and surface disinfested (B) and seeds dehulled, surface disinfested, and exposed to plant material inoculum (C).

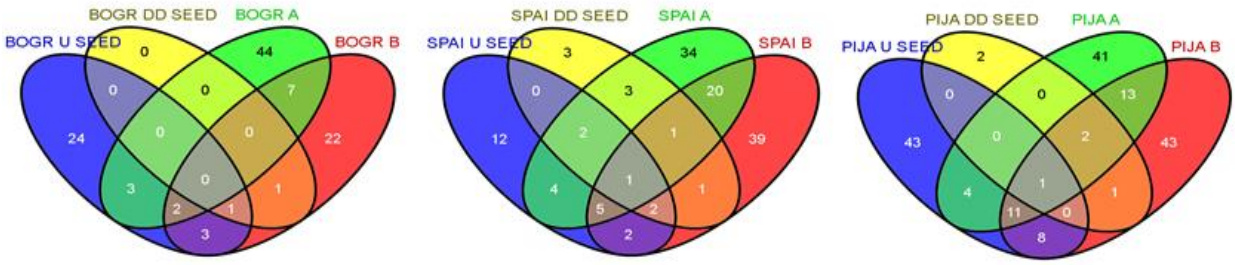

Figure 7. Genus level taxonomic similarity comparison between the bacterial populations in the untreated seed (U), dehulled and surface disinfested seed (DD) and the plants obtained from U (Trmt A) or DD (Trmt B).

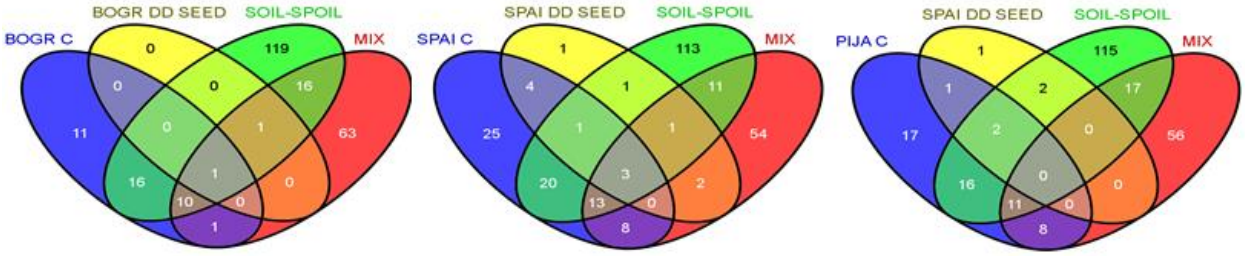

Figure 8. Genus level taxonomic similarity comparison between the bacterial populations in the dehulled and surface disinfested seed (DD), the fallow soil-spoil control treatements, the plant mix used as soil inoculum (MIX), and the plants grown from DD seed exposed to the MIX (Trmt C).

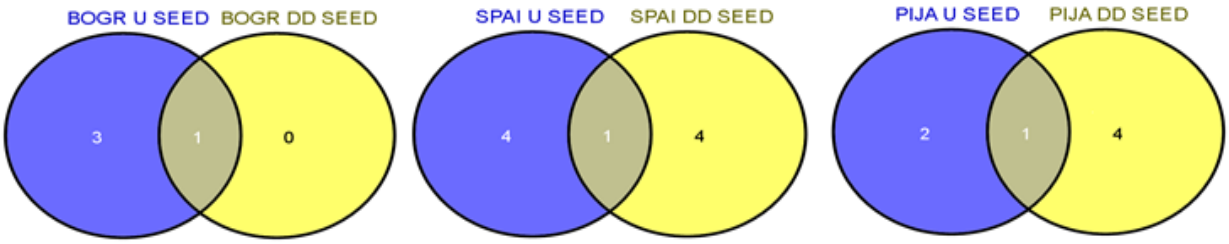

Figure 9. Evidence for seed and seed hull fungal taxonomic putative specificity or similaritiy. Fungal populations of untreated seeds (U) compared to dehulled and surface disinfested seeds (DD). Note that a minority of taxa were perceived unique to DD seeds, likely due to extraction and sequencing bias associated with low abundance taxa.

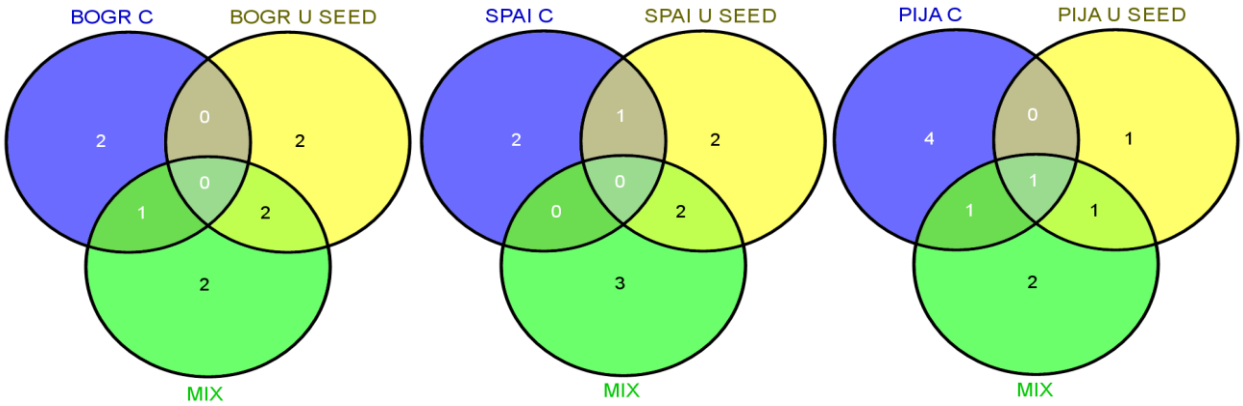

Figure 10. Genus level taxonomic similarity comparison between the fungal populations in the untreated seed (U), plants grown from dehulled and and surface disinfested seed (DD) and exposed to plant material inoculum (Trmt C), and inoculum mix (MIX).

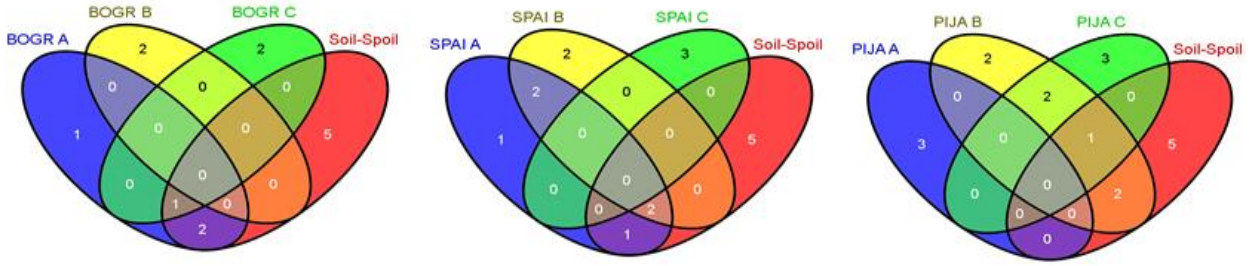

Figure 11. Genus level taxonomic similarity comparison between the fungal populations in the fallow soil and spoil control treatments and the populations recovered from plants grown from untreated seeds (A), seeds dehulled and surface disinfested (B) and seeds dehulled, surface disinfested, and exposed to plant material inoculum (C).

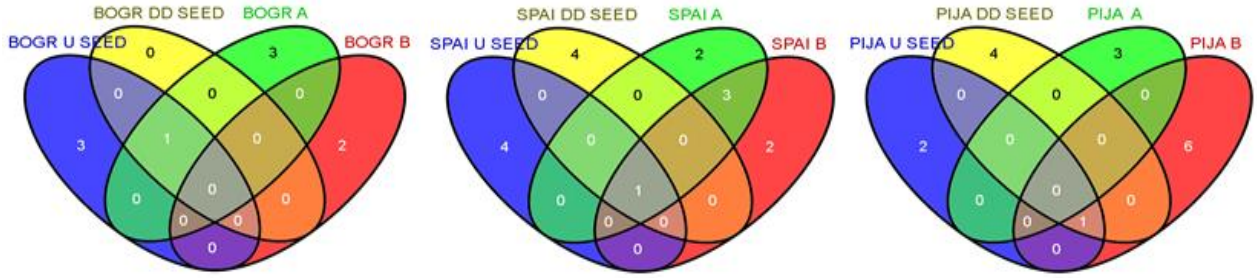

Figure 12. Genus level taxonomic similarity comparison between the fungal populations in the untreated seed (U), dehulled and surface disinfested seed (DD) and the plants obtained from U (Trmt A) or DD (Trmt B).

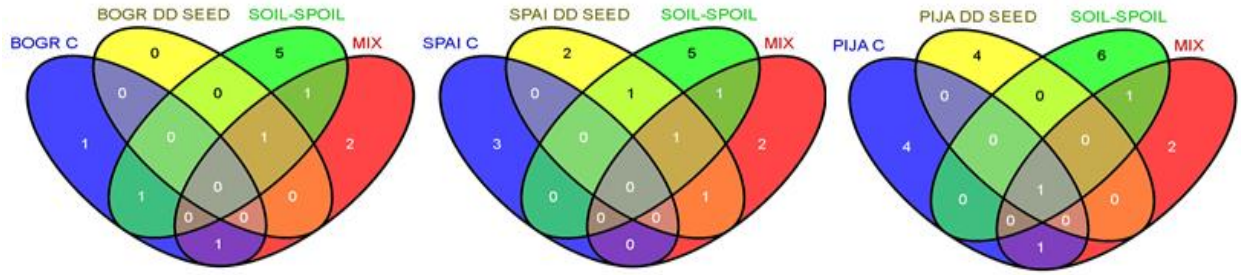

Figure 13. Genus level taxonomic similarity comparison between the bacterial populations in the dehulled and surface disinfested seed (DD), the fallow soil-spoil control treatements, the plant mix used as soil inoculum (MIX), and the plants grown from DD seed expsoed to the MIX (Trmt C).

Discussion

All plant species were exposed to the same ground plant material, allowing reasonably equal opportunity for interactions with the same set of introduced microorganisms (Fig. 2 and Fig. 3). It is shown that all tested species, under each treatment, contained microbial consortia, thought to be cryptic endophytes within the dehulled disinfested seed (Lucero et al., 2011), and that all soil and

spoil contained certain microbial consortia (Fig. 4 and Fig. 9 seed associated microbial consortia; Fig. 6 and Fig. 11 soil and spoil associated microbial consortia). Untreated seed (Trmt A) potentially contained additional endophytes within the seed hull and also epiphytes (Fig. 4 and Fig.9) (Gallery et al., 2007; Hardoim et al., 2012). Plants grown in Trmt B were only subject to influence by any cryptic endophytes likely contained in the dehulled and surface disinfested seed and microbial consortia contained in the soil and spoil (Fig. 7 and Fig. 12 and Fig. 6 and Fig. 11). Microbial consortia identified in the ground plant mix used in Trmt C (Fig. 2) are thought to have contained a mix of epiphytes and endophytes (Porras-Alfaro et al., 2008; Rodriguez et al., 2008; Porras-Alfaro et al., 2011). Trends in plant growth for each species are inherently linked to plant genotypes, but differences in plant growth parameters between treatments of a single species indicates a possible effect of the inoculation and seed treatment (dehulled and disinfested) on plant growth. This may be hypothesized to be due to the recruitment (horizontal transfer) of microbial consortia (Saikkonen et al., 2004; Posadda et al., 2007; Gallery et al., 2007; Kluger et al., 2008) from ground plant material, recruitment of microbial consortia from the soil and spoil (Gallery et al., 2007; Kluger et al., 2008; Khidir et al., 2010; Knapp, 2012), the absence of endophytes contained in the seed hull, and interactions among microbes of different origin (Mukerji et al., 2006). In addition, it is unknown to what extent chemical compounds potentially contained in the seed hull or in the inoculum inhibit, or otherwise affect, plant growth (Kefeli and Kadyrov, 1971; Kremer, 1987; Facelli and Pickett, 1991).

Evidence of these processes was suggested by the diversity profiles in the various sample matrices and across treatments. Fallow soil-spoil taxa were also identified in plant tissue from Trmt A, B, and C. The taxa common between soil-spoil and plant tissue was unique from taxa found in untreated seed, dehulled-disinfested seed, or from the MIX. MIX taxa was partially common to taxa from Trmt C plant tissues and distinct from the community identified in fallow soil-spoil, untreated seed, dehulled-disinfested seed, and plant tissue from Trmt A and B.

Evidence of microbial exclusion by seed treatment was shown (Fig. 4 and Fig. 9). Certain taxa could only be identified in the dehulled and disinfested seeds (Fig. 4). This was likely due to their relatively low abundance, having been obscured in the non-treated seeds by the relatively more abundant general epiphytes and hull associated microbes.

Chemical analysis of seed and inoculum was not conducted. Seed germination rates (Table 3) between untreated and dehulled-disinfested seed supports the possibility that the presence or

absence of seed hull associated factors, either chemical compounds and/or endophytes and epiphytes, may affect seed germination. However, seed treatment only (Trmt B) did not significantly change plant growth (Table 3 and Fig. 11). Seed treatment and exposure to inoculum (Trmt C) led to enhanced growth of *S. airoides*.

Note that plants were under no water stress and were grown under controlled temperature and light conditions to mimic initial mine remediation conditions. Plants did not exhibit any symptoms of pathogen or insect damage. Soluble salts and Na were high in soil and spoil, but not sufficient to impede plant growth (USDA-NRCS, 2012a,b,c). The spoil layer did not inhibit root growth, and roots proliferated in spoil regardless of species or treatment. Available soil N and other macronutrient contents were low, but adequate for these rangeland species (USDA-NRCS, 2012a,b,c). Plants did not exhibit symptoms of nutrient deficiencies or mineral toxicity.

Seed dehulling and disinfestation had variable effects on germination rates among plant species. Effects of seed treatment differed between species. Seed dehulling and disinfestation enhanced germination rates for *P. jamesii* while it had no measurable effect or was correlated to a decrease in germination rates for the other two species (*S. airoides* and *B. gracilis*) respectively (Tables 3 and 5). *S. airoides* plants grown from untreated seed (Trmt A) outperformed plants grown from dehulled-disinfested seed indicating loss of function associated with the seed hull. Taxonomic comparison of microbial genera associated with untreated seed and disinfested-dehulled seed indicate a 53.6% reduction in bacterial genera and an 80% change in fungal genera because of the seed treatment (Fig. 4 and Fig. 9). For *S. airoides* (Trmt C) seed exposure to the plant material mix had a positive effect on measured plant growth parameters. Seed exposure was associated with accelerated growth, taller plants and larger shoot mass, root mass, and root volume. Taxonomic comparisons for the *S. airoides* Trmt C indicates that *S. airoides* (Trmt C) plants shared fungal and bacterial genera unique to MIX or the fallow soil-spoil. Lucero et al., (2008a) propose that endophytic microbes present in tissues of one plant may colonize a different plant species when plant materials are in direct contact with one another. As all measured growth parameters were enhanced, it might be hypothesized that 1) multiple endophytes were recruited (Herrera et al., 2010; Khidir et al., 2010) each influencing distinct processes of plant organ development (Gasoni et al., 1997; Malinowski et al., 1999; Yuan et al., 2010) or 2) at least a single taxon that might influence specific processes of plant development or work in tandem to improve host fitness was recruited (Waller et al., 2005; Sherameti et al., 2005; Osuna-Avila et al., 2012). Trmt C results

have shown evidence that may support the hypothesis of recruitment of multiple taxa from MIX and from soil and spoil. It is important to note that native *S. airoides* plant material was one of the species also used in the plant material mix. Statistically similar germination rates between dehulled and disinfested seed and untreated seed does not support the seed hull as having an effect, at least initially. *S. airoides* was the only species where MIX-exposed plants outperformed non-exposed plants.

For *B. gracilis*, MIX exposure was positively correlated to plant height and root length, but growth rate, root volume, root mass, and shoot mass were not obviously enhanced. Disinfestation of seed, regardless of the exposure status, (i.e., Trmts B and C) led to longer roots and taller and more slender plants but was negatively related to germination efficiency. These results suggest a possible role for hull associated factors. Barrow et al. (1997) demonstrated such a functional relationship between *A. canescens* and hull associated microbes indicating their importance in plant establishment and survival. Seed treatment decreased seed bacterial and fungal diversity by 93.9% and 75%, respectively. Although Trmt C *B. gracilis* shared fungal and bacterial taxa with the MIX or fallow soil-spoil, this did not necessarily associate with any notable changes in the plant growth parameters we monitored.

For *P. jamesii* seed exposure to MIX was correlated with depressed plant growth. Inhibition of plant growth and development in the exposed treatment may be due to selective negative functions, e.g. pathogenic microbial consortia, contained in the MIX. Seed treatment did also not produce any notable changes in plant growth parameters although it enhanced germination.

Conclusion

We conclude that both removing the seed hull and employing plant residues (i.e. MIX), as presented here, can have functional impacts on plant germination and development and that such impacts will vary by plant species. Such differences in plant response to dehulling and/or exposure to the plant material mix suggests specificity of seed associated factors, differential interaction between plant and environmentally induced functions, and specific plant responses to factors associated with the plant material mix. Nutrient effects associated with the plant material mix are assumed insignificant due to the small amounts of inoculum utilized. Soil and spoil disturbance during the setup of the experiment was uniform across the treatments and was therefore not expected to affect differentially any of the treatments. Growth differences observed across treatments indicate complex and potentially beneficial interactions between growing plants and

external plant material. These interactions, which may be hypothesized to be mediated by seed hull or other plant associated microbes, should be understood and considered so that they may be fully leveraged to enhance the efficiency of arid land mine restoration. Further analyses, at sequence level identity, of microbial diversity in collected plant tissue, soil and spoil will help to verify the dynamics of microbial transfer between soil, host plant, and any donor plant. Moreover, a field planting experiment that utilizes planting units inoculated with the described inoculum pre-field-planting would allow for the elucidation of the effect of inoculation on plant growth parameters under natural stress conditions. Cover, initial survival rate, reproductive potential, and plant tissue secondary metabolite concentration could be measured throughout growing seasons to evaluate the role of any exposure to a source of microbial epiphytic and endophytic consortia on plant growth and development in field conditions.

Acknowledgements

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