INTEGRATION OF COAL MINE TAILING AND MYCORRHIZAL FUNGI TO ASSOCIATE *LOLIUM PERENNE* AND *POA PRATENSIS* SEED GERMINATION AND GROWTH PERIOD

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ABSTRACT

Coal mine tailing can be a new source as an organic fertilizer for crops and grass. It also plays a vital role in seeds germination and also promotes the growth of lawn grasses. The aim of this study is to explore the effects of coal mine waste as well as mycorrhizal inoculum association on germination and morphological traits of seedlings of perennial ryegrass (*Lolium perenne* L.) and Kentucky bluegrass (*Poa pratensis* L.) lawn varieties. The germination of seeds was conducted on coal mine tailing and a mixture of coal mine tailing and mycorrhizal fungi. The results showed that coal mine with mycorrhizal fungi enhance the germination capacity, the length of seedling shoots and roots, number of roots and dry mass of shoots and increased mean germination time (MGT) of seeds. The results of this study indicate that germination capacity of *P. pratensis* varieties was modified by coal mine tailing mixed with mycorrhizal fungi. Results indicated root dry mass in *Lolium perenne* grown in coal and mycorrhizal association was characterized by high shoot and root contents of N and P, whereas plants grown on only coal mine tailings had high shoot and root contents of Ca and Al.

KEYWORDS:
Coal mine tailing, mycorrhizal fungi, *L. perenne* bluegrass, *P. pratensis*

INTRODUCTION

Perennial ryegrass (*Lolium perenne L.*) and Kentucky bluegrass (*Poa pratensis L.*) are the most widely used in Ege region as lawn grasses in private and commercial areas such as sport stadiums. This study aimed to seek factors and parameters on growth and productivity in emphasized species. Therefore, these grass species applications can be extensively used in most of landscape and sports facilities as well as home lawns, sports fields and urban green areas as well if they are properly utilized [1]. Many environmental factors, particularly the hot climate and low input nutrient soil are the main factors limiting the germination of seeds and development of plants.

In Ege region within Turkey, in these conditions the use of natural resources like coal mine tailings as a less expensive materials and soil beneficial microorganisms such as mycorrhizal fungi is frequent and high [2, 3]. It is also known that the coal mine can release organic matter in soil and provide a condition which is subjected to microbiological-biochemical processes of transformation. In response to these challenges, this study tests the growth potential of two commercial grass in Turkey alongside using coal mine tailing the following hypotheses were tested: Perennial ryegrass and Kentucky bluegrass can be successfully established and produce viable yields in coal mine tailing soils after the summer growth period of growth following preliminary reclamation measures prior to planting. Some heavy metals as Cu can also better accumulated in both grasses with mycorrhizal fungi association in both plant roots. However, studies addressing the influence of mine waste on seed germinations in natural sites are rare. An increase in the macronutrient concentration in surrounding environment has often led to the production of seeds that are heavier and have greater quantities and nutrient concentrations [4].

MATERIALS AND METHODS

Mine tailings materials. Mine waste or mine tailings was provided from an active coal mine sector in Bayindir, EGE region in south western of Turkey. The elemental analysis of the mine tailing is as presented in Table 1.

Biological materials and morpho-physiological measurements. Seeds and plant preparation: a greenhouse experiment was conducted using planted material such as seeds of Perennial ryegrass (*Lolium perenne* L.) and Kentucky bluegrass (*Poa pratensis* L.). The study evaluated the effects of coal mine

TABLE 1
Content and elements analysis of coal mine tailing used in the experiment

Analysis	Unit	Result	Detection limit	Recovery	Level
Al	mg/kg	19.08	0.17	88.9	High
Cu	mg/kg	0.53	0.05	101.5	Low
Zn	mg/kg	34.62	0.05	94.89	High
Fe	mg/kg	60.52	0.05	61.72	Very High
P2O5	mg/kg	30	0.06	98.87	Sufficient
Ca	mg/kg	5227	0.1	115.85	high
Mg	mg/kg	21.4	0.05	100	Very low
Mn	mg/kg	11.87	0.05	149.58	Sufficient
K2O		2.7	0.03	97.18	Very low
Organic carbon	%	1.85			Low
pH		7.5			Slight acetic
Salinity EC	%	0.01			Non-saline
CaCO3	%	2.7			Low
Total N	%	0.09			Average

waste on the germination and seedling growth of perennial ryegrass (*Lolium perenne* L.) and Kentucky bluegrass (*Poa pratensis* L.). Research was carried out in 2014 in two separate experiments (coal with mycorrhizal fungi and coal without mycorrhizal fungi) repeated twice and totally 3 replications based on AVONA statically program in different conditions of germination. The analysis of seed purity was performed according to ISTA Rules (2014) and for the seeds of *P. pratensis* the uniform blowing method was applied. Seed purity of *L. perenne* varieties was 99.1–99.7% and a 1000 seed mass varied from 1.182 to 1.325g. Purity of *P. pratensis* seeds was 97.9–98.3% and a 1000 seed mass was 0.310 and 0.402g respectively. Seeds were initially treated with a 70% solution of ethanol for 2 minutes for surface sterilization, rinsed with distilled water three times, and then quickly dried with a paper tissue. Seed germination conditions were established according to ISTA Rules (2014).

Germination according to the ISTA Rules (2014). Germination tests to assess germination capacity of investigated varieties were carried out in accordance with different temperature and light conditions: 35/20C (day/night) temperature regime and 8/16h (day/night) photoperiod under cool, white light 20Wm-2. Three replicates of 50 seeds of each variety were placed in a square plastic box (capacity 0.8 l) containing three layers of filter paper (65 g m-2) moistened with distilled water (60% compared to control volume of the water).

Germination under mycorrhizal and without mycorrhizal associations. Germination capacity was estimated in constant temperature 35/25°C (day/night), white light 20 W m-2 and variable photoperiod 12/12h (day/night). Three replicates of 50 seeds of each variety were placed in plastic boxes containing three layers of filter paper moistened in solution of 6 million spore of (*Glomus mossae*).

Mean germination time (MGT) under stress conditions. The study was carried out in analogous drought stress levels at the constant temperature of 35°C under exposure to dark conditions (0/24h photoperiod) [5]. Three replicates of 50 seeds of investigated varieties were germinated on 10-diameter Petri dishes laid with two layers of moistened filter paper. To avoid seed float on the surface of the solution, the filter paper was planted into plastic disc with a thickness of approx. 3mm. In order to prevent the evaporation during germination of seed, in each experiment the boxes and Petri dishes were put into airtight transparent plastic bags. Measurements. Germination capacity data were collected based on the final counts of normal seedlings after 10 days for *L. perenne* and 21 days for *P. pratensis*. Seedlings were scored as normal as defined by ISTA Rules (2014) and Handbook of Seedling Evaluation (2013), as type D group A 1-2-3-1. MGT in both stress conditions was determined every day from the beginning to the end of germination. In stress conditions, because of slow germination of seeds, it was conducted over a longer period than foreseen in the Rules of ISTA. The number of germinated seeds was determined, considering the seeds with radicle 2mm long. Then the germinated seeds were removed from Petri dishes. The mean germination time was calculated on the basis of the number of germinated seeds as follows [5]: MGT (day) = $\sum (fx)/\sum f$, where x is the day of counting, f – the number of newly germinated seeds on each day. To estimate morphological parameters of tested varieties, twenty normal seedlings were sampled randomly from each plastic box in the end of study, according to the ISTA Rules (2014) and drought conditions. The length of shoots and roots of seedlings as well as the number of roots was determined. The distance from the crown to leaf tip and root tip was measured as the shoot and root length, respectively. Then the seedlings were separated into roots and shoots and their dry mass was evaluated after drying in an oven at 80C for 24 h to a constant weight. The obtained results were expressed per seedling (mg per plant); the ratio of root to shoot (R:S) mass was also estimated.

Determine root mycorrhization rate [6]. Determination of mycorrhization and they were kept in 70% ethanol at -20°C until the staining. Before the staining, roots were packed in tulle and washed with 1%HCl for one minute and then distilled water for one minute, and the process was repeated for three times. The washed and packed roots were put into the falcon tubes that were filled with 10% KOH solution (w/v) and kept in water bath at 60°C for 4,5 hours. The packed roots in the tubes were transferred to new 50 ml falcon tubes which were filled with 0.05% (w/v) trypan blue solution (1:1:1 lactic acid, glycerol). Two root samples were taken from each of the pots for the determination of mycorrhization and they were kept in 70% ethanol at -20°C until the staining. Before the staining, roots were packed in tulle and washed with 1%HCl for one minute and then distilled water for one minute, and the process was repeated for three times. The washed and packed roots were put into the falcon tubes that were filled with 10% KOH solution (w/v) and kept in a water bath at 60°C for 4,5 hours. The packed roots in the tubes were transferred to new 50 ml falcon tubes which were filled with 0.05% (w/v) trypan blue solution (1:1:1 lactic acid, glycerol, and water) and kept in dark for one week. The packed roots were opened and aligned on glass slides. Cover slides were closed on the other slides and fixed by transparent nail polish. The slides were observed under the microscope. 30 fields of vision that the object occupies were observed for each slide and mycorrhization counts were done based on these 30 fields for each sample. Microscope Axio Observer.Z1, with EC Plan-Neofluar 20x objective, and AxioCam MR5 camera was used.

Statistical analysis. The presented data are the mean values from experiments repeated twice. Comparisons of means were performed on the basis of two- way analysis of variance and multiple comparisons based on LSD. Pearson's correlations were used for evaluation of relationships between pairs of variables. The analyses were conducted using statistical program Statistica, version 12 (StatSoft Inc., USA). For all analyses, significance level was set at $P \leq 0.05$. For the evaluation of relationships between all traits across cultivars and treatments, principal component analysis (PCA) was conducted. The results of PCA were presented as a biplot of the first and the second principal components (PC1 and PC2).

RESULTS AND DISCUSSION

Effect of coal mine tailing on seed germination. Germination capacity of *L. perenne* varieties in conditions without coal mine waste was similar: ranged from 87% to 96%, according to ISTA Rules (2014): day/night 35/20C temperature regime and 8/16 h photoperiod, as well as at constant temperature (25^{o}C) in the dark and also with 12h exposure to light. At the same time, the obtained results show that seed germination of *P. pratensis* varieties was highly affected by light. Our previous studies have demonstrated positive effect of longer day conditions on seed vigor of *P. pratensis* varieties, while the germination capacity varied [7, 8]. According to germination study [9], germination tolerance for light intensity is expected for some grass species in natural environment where seeds could be exposed to different light regimes, ranging from full light to dark. In coal mine conditions the two investigated species had also different response to coal mine contents with high levels of C and moderate heavy metals such as Cu, Zn and Pb. *L. perenne* showed a decrease of seed germination only at the highest level of coal mine tailing (100% coal mine tailing without mycorrhizal fungi) characterized by low germination in this situation (approx. 69%) demonstrating. It is in agreement with the previous study [10] who stated that *L. perenne* was relatively tolerate to high C and certain levels of heavy metals in sunflower. Our results have been confirmed in the comparable studies [11] found that coal mine tailings caused 30% reductions in germination in the turfgrass varieties of *L. perenne*. Some other study found out strong inhibition of the germination of *L. perenne* seeds mine tailings conditions as well [12]. According to the results of our study, *P. pratensis* were less tolerant to high C and heavy metals concentrations than those of *L. perenne*. Using mycorrhizal fungi and inoculate it to the seeds showed significant difference between coal mine with and without mycorrhizal treatments. This is in agree with the recent study [13] on *L. perenne* and *F. arundinacea*: increased plant biomass and root metal concentration; decreased shoot metal using coal mine tailing as organic matter for growth promoting.

Plant dry biomass. Shoot dry mass of *Lolium perenne* was significantly greater than that of *Poa pratensis* in coal mine tailings treatment (Figure 1). Although *P. pratensis* had a slightly higher root dry mass, there was no significant difference in root dry mass between the two plant species in coal mine tailings. Shoot dry mass of *L. perenne* increased significantly in the order coal mine tailings and AMF associations. Root/shoot ratio of *L. perenne* was significantly lower in coal mine tailings treatments without AMF inoculum presences than coal mine tailings with AMF application. In coal mine without AMF association *L. perenne* had a slightly but not significantly lower root dry mass than in the lignite layers. There was no significant difference in total root dry mass of *L. perenne* in coal mine tailings compared to *P. partensis* grown in coal mine tailings with non-AMF.

Total root length and root length/root dry mass ratio. *L. perenne* showed a higher root length than *P. partenis* in mine tailings with and without AMF inoculation (Table 2). Root length of *L. perenne* was greater in two substrates coal and AMF than in the other two substrates. Root lengths of *L. perenne* in coal and without AMF were very low in both *L. perenne*. and *P. partenis*. The root length/ root dry mass ratios showed a similar pattern as root length (Table 2).

Shoot dry mass of *L. perenne* increased significantly in the order coal and AMF associations and the following parameters as such Root/shoot ratio of *P. partenis* was significantly lower in coal and control treatments as compared to *L. perenne* in coal with and without AMF treatments. *P. partenis* showed a slightly but not significantly lower root dry mass in the control similar to *L. perenne*. However, there was significant difference in total root dry mass of *P. partenis* in coal and AMF compared to *L. perenne* grown.

Total root length and root length/root dry mass ratio. *L. perenne* indicated a higher root length than *P. partenis* in only coal and coal with AMF association (Table 2). Root length of *P. partenis* was greater in coal with AMF than in the other two substrates. Root lengths of *P. partenis* in coal and control substrates were very low. The root length/root dry mass ratios showed a similar pattern as root length (Table 2).

Element content of shoots and roots. The shoot N content was greatest in *L. perenne* in coal application with AMF inoculation. Besides, shoot S and Mg was also higher in coal and coal plus AMF in *L. perenne*, compared to *P. partenis* in the same treatments. Also *P. partenis* treated with coal and AMF shown lower values of S, Mg than the *L. perenne* in the same applications. (Table 3). In the coal mine tailings application with AMF, the shoot P content was significantly higher in *P. partenis* than *L. perenne*. Moreover, *P. partenis* preformed higher P and lower Mg contents than other treatments ($P \leq 0.1$). Along the element's accumulations shoot K content was lowest in *L. perenne*. Shoot Ca content in *P. partenis* was was significantly higher compared than *L. perenne* in coal with AMF and slightly, but not significantly higher than in *P. partenis* with only coal treatment. (Table 3). Root N content of *L. perenne* was significantly higher in coal mine tailing with AMF than *P. partenis* in coal mine and AMF and only coal treatments. Magnesium, Ca, and Al contents were highest in roots of *L. perenne* in coal mine tailings and AMF. However, this difference was significant for Mg, Ca, and Al only compared to *P. partenis* in coal mine tailing without AMF, and for Ca and Al compared to *P. partenis* in cola with AMF. In coal mine with AMF inoculum, root Ca content was twice as high in *L. perenne* than in *P. partenis* without AMF($P \leq 0.1$). Root Ca content was significantly higher than shoot content in *L. perenne* whereas no significant difference between root and shoot Ca content was observed in *P. partenis* in this substrate (without AMF). Ca/Al and Ca+Mg+K)/Al ratios were highest in the shoots of *L. perenne* in coal mine with AMF association, especially in roots (Table 3). In coal mine tailings with AMF, the root Ca/Al ratio in *L. perenne* was twice as high than in *P. partenis* (Table 3).

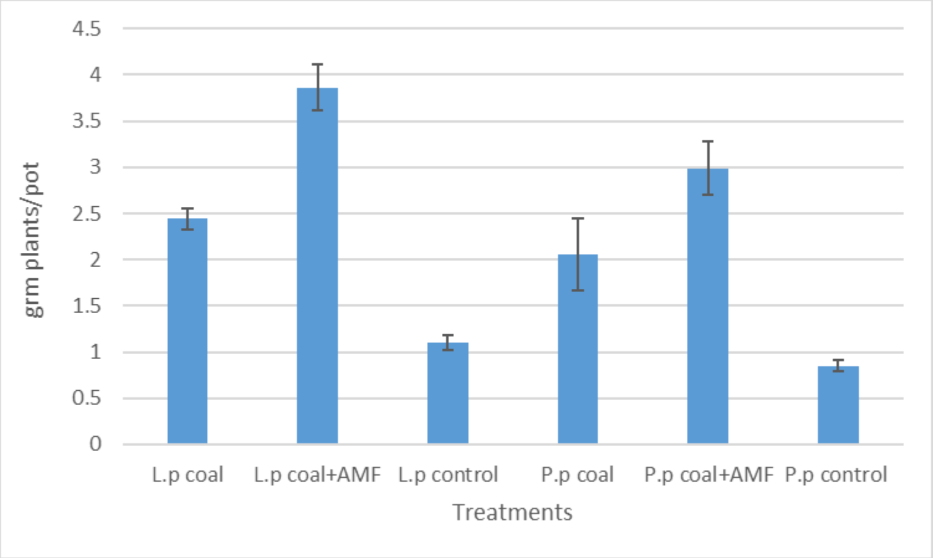

FIGURE 1
Plants dry matter in different treatments with and without mycorrhizal fungi (Pr > F: 049).**

TABLE 2
Visible root length (median) of *L. perenne* and *P. partenis* on the front side of the rhizotrons and ratios of root length/root dry mass after 3 months of growth in different substrates: coal mining with and without AMF

Treatments	Root length (cm)	Root length/root dry mass	Root mycorrhizations rate (%)
L. perenne **control**	12.55	54.01	Not observed
L. perenne **coal and AMF**	29.78	105.87	66.77
L. perenne **coal**	20.41	85.21	Not observed
P. partenis **control**	10.02	40.32	Not observed
P. partenis **coal and AMF**	15.63	128.77	41.69
P. partenis **coal**	12.47	55.29	Not observed

TABLE 3
Comparison of elements accumulations in both plants shoot and root coal mine with and without AMF associations

Treatments (mg/g)		C	N	S	P	Mg	K	Ca	Al	(Ca+Mg+K)/Al	Ca / Al
Shoots	*L. perenne control*	152.11	13.9	6.7	1.2	3.21	8.41	11.58	1.91	6.06	19.19
	L. perenne coal	321.2	14.2	7.8	1.3	4.55	10.19	13.21	1.08	12.23	25.87
	L. perenne AMF	521.2	24.4	9.2	3.2	6.10	14.80	18.71	1.37	13.65	39.06
	P. partenis control	189.5	9.7	3.9	2.1	2.33	10.85	2.87	1.09	2.63	15.85
	P. partenis coal	385.3	10.5	4.1	3.8	2.56	12.33	5.08	1.41	8.56	23.45
	P. partenis AMF	285.3	18.9	5.1	6.7	3.87	15.52	15.06	1.55	9.71	28.10
Roots	*L. perenne Control*	190.2	8.7	1.2	0.55	1.01	0.18	10.28	0.45	22.84	12.70
	L. perenne Coal	222.3	9.2	2.6	0.81	1.25	0.28	16.85	0.51	13.43	14.96
	L. perenne AMF	402.3	15.9	5.0	1.38	1.99	2.99	9.92	0.63	15.74	20.47
	P. partenis control	190.5	6.1	1.4	1.6	0.55	0.21	3.74	0.71	5.26	4.72
	P. partenis Coal	255.4	7.3	2.9	2.9	0.99	0.51	5.03	0.88	5.71	7.42
	P. partenis AMF	311.8	8.8	3.5	0.69	1.23	0.68	7.77	0.91	8.53	10.44

DISCUSSION AND CONCLUSION

This study showed that (i) growth of *P. partenis* in only coal mine tailing was poorer than in medium with AMF and (ii) *L. perenne* grew better *P. partenis* in coal mine tailings and AMF association. This indicates that *L. perenne* can cope better with the adverse soil conditions in the overburden material than *P. partenis*. The medium differed in nutrient concentration in the equilibrium soil solution which may be explained by differences in total nutrient content as well as pH among the substrates (Tables 1 and 2). In the coal mine tailings, the equilibrium soil solution was characterized by high SO4 $^{2-}$, Ca, Al, Mg and low NO^{3-} concentrations. Shoot dry mass of *L. perenne* in coal mine with AMF was higher than in the other two substrates (Figure 1), which could be due to the higher contribution of AMF to uptake nutrient availability in this substrate. In agreement with this hypothesis, the high N concentration in the equilibrium soil solution in the coal mine tailings (Table 2) was associated with an increased N content in shoots and roots of *L. perenne* in coal mine tailings (Table 3). *P. partenis* in coal mine with AMF also had higher shoot and root P contents. *P. partenis* coal mine tailings had the lowest shoot and root dry mass which may be explained by the lower N concentration in the equilibrium soil solution of this substrate. Whereas the N content of *L. perenne* grown on control and only coal mine tailings was 13.8mg g-1 shoot dry mass, *L. perenne* in coal mine plus AMF showed higher shoot N content (24.4mg g-1) shoot dry mass, which is, however, still considered adequate for grassland plants [10,11, 13, 14, 15]. The Al concentration in the equilibrium soil solution of the coal mine tailing was high (Table 2). High Al concentrations can inhibit root growth [16] and reduce the uptake of nutrients such as P, Ca, K and Mg [17]. However, root dry mass was not significantly lower in *L. perenne* grown on coal mine in addition with mycorrhizal fungi than in the other such as control and only mine tailings. This may be explained by the high (Ca+Mg+ K)/Al ratio in the equilibrium soil solution of this substrate (Table 2). According to similar study root growth of *L. perenne* is not affected at Ca/Al ratios [12]. In the coal mine mixture with mycorrhizal fungi Ca/Al ratios in control and only mine tailings, equilibrium soil solution, and roots were always > 1 (Tables 1, 2 and 3). Some study reported that growth medium using mine and AMF (mycorrhizal fungi) contain high amounts of Ca, not only as a result of amelioration but also as a consequence of relatively Ca-rich parent material [15]. The high Ca concentration obviously plays an important role for plant growth in these acidic substrates. Shoot dry mass of *L. perenne* increased significantly in the following order: control > coal mine tailings > coal mine mixture with mycorrhizal fungi. In the medium coal mine nutrients and water would be freely available, but also leached easily. The coal mine tailings would serve as buffer zones with low but more stable nutrient and water availability due to sorption of water and nutrients to the coal mine tailings. Roots grew into the coal mine tailings mixture with mycorrhizal fungi, which had higher Ca and lower Al content than the mine tailings (Table 1). Data showed that cola mine and mycorrhizla fungi combination can be used as a nutrient and water reservoir by *L. perenne*. Therefore, in the present study, roots appeared to grow freely through the coal mine tailings. Hydrophobicity of the lignite, as it was shown in some mine tailings in southern Turkey defer for old organic materials [18]. In the present study the rhizotrons were watered continuously; thus, hydrophobicity of the lignite may not have been strongly expressed. In the mine tailings, the Ca content in shoots of *P. partenis* was lower than in *L. perenne* (Table 3). This might be due to the increased shoot dry mass of *P. partenis* leading to a dilution effect for Ca. Another reason could be the different strategies of the two grass species concerning the distribution of Ca in the plant. In the present study, the ratio of Ca in shoots to Ca in roots was 1.88 *P. partenis* and 1.95 in *L. perenne*, suggesting that *P. partenis* accumulates Ca in the roots and translocates less into the shoots than *L. perenne* (Table 3). recent research also found a strong accumulation of Ca in roots of *P. partenis* compared to various dicots [12]. The higher root Ca content in *P. partenis* lead to a higher Ca/Al ratio in the roots, thus reducing the negative effect of high root Al content. This may contribute to the significantly higher shoot dry mass of *P. partenis* on mine tailings mixed with mycorrhizal fungi compared to *L. perenne* on the same substrate. The high root dry mass (Figure 1) and high root length (Table 3) may allow *P. partenis* to explore the lignitic substrate more intensively and use nutrients and water more efficiently than *L. perenne*. A different root growth behavior is also apparent in the disparity between root dry mass and root length. A higher root length/root dry mass ratio was observed for *L. perenne* than for *P. partenis* in coal mine tailings and mycorrhizal fungi combination (Table 3). Root length was determined only along the front side of the rhizotrons whereas root dry mass included all roots in the rhizotrons. The higher root length/root dry mass ratio of *L. perenne* could therefore be due to a greater gravitropism of its roots resulting in more roots growing downwards along the front plate compared to *P. partenis*.

This study showed that growth of *L. perenne* seedlings during their week year was successfully grew in mine tailing containing Mycorrhizal fungi compared to sand (control) and only mine tailings from a mine site, suggesting that *L. perenne* is well-adapted to the mining substrate and can take advantage of nutrients and water stored in the lignite. On the other hand, growth was improved when mycorrhizal fungi additionally added in the mine tailings. *L. perenne* can utilize nutrients and water stored in lignite existed in coal mine tailings. *P. partenis* grew weaker in the mining substrate than *L. perenne* indicating that *P. partenis* may be a better choice for reforestation of mine overburden containing lignite.

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