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Germination and seedling growth of *Moringa oleifera*, *Moringa stenopetala* and *Phaseolus vulgaris* grown in polluted soil amended with coal fly ash

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Abstract

A germination test was carried out to identify plants that can germinate and survive in polluted soil (with and without ash) collected 2.5km east and 2.5km west, 20km west and 55km west (control) of the BCL Cu/Ni mine smelter in Selebi-Phikwe, Botswana. The experiment was carried out using *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala*. Soil acidity and heavy metal stress reduced germination percentage, coefficient rate of germination, root and shoot growth and dry weight, root: shoot, vigour index and tolerance index of all species. Percentage reduction followed the order 2.5km west < 20km west < 2.5km east < 55km west. *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* germinated in all soils. Their ability to germinate in polluted soil indicates tolerance to heavy metal and soil acidity stress and so they have potential for use in phytoremediation of polluted soils around the mine. *Phaseolus vulgaris* had the highest overall germination performance but there was no significant difference between the *Moringa*s. Application of coal fly ash increased all the germination parameters and so coal fly ash has potential for use in amending polluted soil around the mine for phytoremediation purposes.

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Introduction

Worldwide, soil is being seriously degraded as a result of mining and smelting. The undesirable accumulation of toxic heavy metals due to mining and smelting not only causing worldwide devastation of agricultural soils and products but also pose serious food safety issues, health risks and disruption of ecosystems. Exposure to heavy metals produces detrimental effects on plant growth, development and/or other physiological processes (Fargasova, 2001; Heiss *et al*., 2003; Kupper *et al*., 1998; Peralta *et al*., 2001). Mining and smelting in Selebi-Phikwe, Botswana by the BCL Cu/Ni mine has been reported to have resulted in heavy metal soil pollution (Vurayai *et al*., 2015; Ekosse *et al*., 2004) and soil acidity (Vurayai *et al*., 2015) and this has affected the atmosphere, soils, flora and fauna (Ekosse 2003, 2004, 2005,).

Heavy-metal phytotoxicity and soil acidity impairs germination. Seed germination is the first step of a plant's life; it is one of the critical developmental stages in the life of any individual species. Soil acidity and heavy metal toxicity reduce or prevent seed germination resulting in dead zones near the BCL Cu/Ni mine, Selibe-Phikwe, Botswana (Ekosse *et al*., 2005). Bare soil is more susceptible to wind erosion and consequent spread of contamination by airborne dust. Immediate remediation is thus required to reclaim the site by establishing a vegetative cover in order to minimize soil erosion and spread of metal pollution.

Phytoremediation is a promising technology for the clean-up of heavy metal contaminated soils and is an alternative to more expensive remediation technologies because it is a feasible, effective, safe, cheap and non-intrusive technology that uses plants and their rhizosphere to extract, detoxify, and sequester pollutants (organic and inorganic) from soil, sediments, and water (Kranner and Colville, 2011). Plant species are used for extraction, stabilization and / or neutralization of contaminants present in soil (Cunningham and Berti, 2000). Seed germination studies are prerequisite groundwork to determine if plants are suitable for growth on land east and west of the BCL Cu/Ni mine smelter for phytoremediation applications. The ability of a seed to germinate in a medium containing any metal element is a direct indicative of its level of tolerance to this metal (Peralta *et al*., 2001).

The main initial steps in phytoremediation are the identification of species which are able to grow and develop in the contaminated/ polluted soils. The objective of this work was therefore to investigate the effect of soil acidity and heavy metal pollution on the germination and seedling growth of *Moringa oleifera*, *Moringa stenopetala* and *Phaseolus vulgaris* when grown in soil collected east and west of the BCL Cu/Ni mine smelter in Selebi-Phikwe, Botswana. Pollution around the BCL Cu/Ni mine in Selebi-Phikwe, Botswana is said to be related to distance from the mine smelter (Vurayai *et al*., 2015). The current study also determines the effect of distance from the mine smelter on germination performance of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris*.

Vurayai *et al*. (2015) indicated that land east and west of the BCL Cu/Ni mine smelter is acidic and so improving soil physical and chemical properties maybe required for successful phytoremediation of these acidic soils. Various amendments can be used to increase the phytoremediation potential of plants and recent studies have shown that coal fly ash immobilises heavy metals in soil reducing their availability to plants (Su and Wong, 2003; Tsang *et al*., 2014). This effect is attributed to the alkaline nature of coal fly ash which raises the soil pH making heavy metals unavailable and to the coexistence of constituents potentially capable of absorbing heavy metals. The influence of coal fly ash amendment on germination of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris* seeds grown in polluted soil collected east and west of the BCL Cu/Ni mine smelter in Selibe-Phikwe, Botswana was therefore evaluated under greenhouse conditions.

Materials and methods

Seed source

Moringa oleifera, Moringa stenopetala and Phaseolus vulgaris seeds used in the study were collected from the

seed stocks of Department of Agriculture Research, Ministry of Agriculture, and Botswana.

Source of soil and coal fly ash Samples

Soil was sampled from four areas: 2.5km east, 2.5km west, 20km west and 55km west (control) of the BCL Cu/Ni mine smelter in Selebi-Phikwe, Botswana (Vurayai *et al.*, 2015). Samples of coal fly ash were collected from Morupule power station, Palapye, Botswana.

Experimental design

The experiment was arranged in a 2 x 3 x 4 factorial experiment in a completely randomized block design of 5 replications. The treatments were as follows: factor A (Fly ash) which had two treatments (fly ash and no fly ash), factor B (Plant species) which had three treatments (*Moringa oleifera*, *Moringa stenopetala* and *Phaseolus vulgaris*) and factor C (Distance where soil was collected) which had four treatments: 2.5km east of mine smelter, 2.5km west of mine, 20km west of mine smelter and 55km west of mine smelter (control).

Seed germination

This study was undertaken in a greenhouse at University of Botswana, Department of Biological sciences. Coal fly ash quantities were added to soil from each of the 4 sites mentioned above to a final concentration of 7.5% per kg of dry weight. 13cm round and 10.8 cm deep (1 litre) plastic pots were filled with 11 kg soil and 1 seed was planted per pot. The pots were watered to reach 100% plant available water (Rosenthal *et al.*, 1987) every day and the experiment ran for 14 days.

Measurements

Germinated seeds were counted daily for 14 days; seeds were considered fully germinated after the radicals had reached length of 2mm. Germination percentage was calculated according to Iqbal and Rahmati, (1992) and coefficient rate of germination (CRG) was calculated according to Mamo *et al.* (2006).

Germination percent = (number of germinated seeds/ total number of planted seeds) x 100

Coefficient rate of germination = $[\Sigma n / \Sigma (n \times d)] \times 100$

Where

n = number of seeds completing germination on day

d = the time in days starting from day 0; the day of commencement germination test

At the end of the 14 days of the experiment, root and shoot lengths of germinated seeds were measured with a millimeter ruler. The length of shoot and root were recorded by using a centimeter scale.

Tolerance index was then calculated using the root and shoot lengths according to Iqbal and Rahmati, (1992).

Tolerance index = (mean root length of polluted area seedlings/ mean root length of control area seedlings) x 100

Seedling vigour index was also calculated using the method described by Moradi *et al* (2008).

Vigour index = seedling length [root length + shoot length (cm)] x germination percentage

After 14 days, the roots were separated from shoots. Seedlings were subsequently placed in an oven and dried to a constant weight at 60°c and dry weight was measured. Mean weight was calculated and expressed in milligrams (mg). Root: shoot ratio was also calculated.

Statistical analysis

The experiment was repeated twice and pooled data is presented. In-order to determine the effect of treatments, the results were analysed by ANOVA using IBM SPSS Statistics 22. Treatment means were compared using LSD at probability level of 0.05.

Results and discussion

The land east and west of the mine is said to be contaminated with heavy metals (Cu, Ni, Fe, Zn, Mn etc) and is also acidic. Soil pH 2.5km west has been shown to be 3.86, 20km west (4.31), 2.5km east 5.36 and 55km west (6.28) (Vurayai *et al.*, 2015). Germination percentage and coefficient rate of germination of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* were significantly reduced by heavy metal and soil acidity stress (Fig 1; Fig 2).

The dissolution and bio-availability of heavy metals is increased at low pH which increases their availability (Shahandeh and Hossner, 2002). Excess heavy metals reduce germination by inhibiting hydrolysis of polysaccharides, proteins and lipids in cotyledons and endosperm. Heavy metals affect and reduce activity of hydrolyzing enzymes (amylase, proteases and lipases) (Bose *et al*., 1982) and also inhibit respiration by affecting enzymes involved in Krebs cycle (Sheoran *et al*., 1990; Sandalio *et al*., 2001). Nickel (Ni) affects amylase, protease and ribonuclease enzyme activity thus retarding seed germination of many plants (Ahmad and Ashraf, 2012). Heavy metals also inhibit plumule and radical growth thus reducing both germination percentage and coefficient rate of germination.

Phaseolus vulgaris, *Moringa oleifera* and *Moringa stenopetala* were able to germinate successfully in polluted soil collected east and west of the BCL Cu/Ni mine in Selibe-Phikwe, Botswana (Fig 1). Seed germination is the most resistant process to heavy metals (Seregin and Kozhevnikova, 2005). This might be because the supermidermal layers of their seed coats have thickened walls which reduces penetration of heavy metals. The seed coat can be a barrier between the embryo and the environment as it protects the embryo against the heavy metals toxicity (Araùjo and Monteiro, 2005) thus allowing some plants (in this case *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala*) to germinate in polluted soil. The reproductive and aggressive capacity of a species is in fact determined by the percentage values of survival in the natural environments and so the ability of these species to survive on polluted soil shows great potential for their use in phytoremediation.

Root and shoot length, root and shoot dry weight and root: shoot ration of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* were significantly reduced by soil acidity and heavy metal stress (Table 1). Soil acidity is a major growth-limiting factor for plants in many parts of the world (Foy, 1984). Soil pH is a measure of the concentration of hydrogen ions in the soil solution and the lower the pH of soil, the greater the concentration of H^+ ions. It has been suggested that excess H^+ competes with other cations for root absorption sites, interfering with ion transport and uptake, and causes root membranes to become leaky (Foy, 1992) thus reducing root growth which culminates into reduction of shoot growth. Reduction in root and shoot growth will thus result in reduction in root and shoot dry weight and root: shoot.

As mentioned before the dissolution and bio-availability of plant toxic heavy metals is increased at very low pH and so heavy metals are more available in acidic soils like those east and west of the BCL Cu/Ni mine smelter. Excess heavy metals inhibit germination, root elongation and seedling development thus root and shoot length, root and shoot dry weight and root: shoot ration of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* were reduced (Table 1). Roots are the first organs to come into contact with toxic elements and inhibition of root growth appears to be the first visible effect of metal toxicity. This might be because heavy metals reduce both new cell formation and cell elongation in the extension region of the root (Prasad, 1995; Liu *et al*., 2004). Lerda (1992) made similar observations in roots of on *Allium cepa*.

Seedling vigour and tolerance index of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* were significantly reduced by heavy metal and soil acidity stress (Fig 3; Fig 4). Heavy metal and soil acidity stress reduces root vitality and affects the growth process (Cheng 2003). Tolerance index and vigour index are based on the root growth and gives an estimate of the effect of the heavy metals toxicity in the short run (Hakmaoui *et al*., 2007) thus reduction in root and shoot growth in Table 1 resulted in a reduction in both tolerance and vigour index.

Germination percentage, coefficient rate of germination, root and shoot length, root and shoot dry weight, vigour and tolerance index of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* significantly differed ($p<0.05$) according to distance where soil was collected (Fig 1-4; Table 1). They were highest when grown in control soil (55km west) and followed the order

55km west> 2.5km east> 20km west> 2.5km west. This might be because soil pH is considered to be higher closer to the mine smelter (2.5km west) and follows the order 2.5km west (3.86) < 20km west (4.31) < 2.5km east (5.36) < 55km west (6.28). Heavy metal content of Cu, Ni and Fe also followed the same order but vice versa (2.5km west> 20km west> 2.5km east> 55km west) (Vurayai *et al.*, 2015). Heavy metal availability increases at low soil pH and so severity of heavy metal and soil acidity stress thus followed the order 55km west> 2.5km east> 20km west> 2.5km west.

Table 1. Root and shoot length, fresh weight, dry weight and root: shoot of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris* grown in polluted soil treated with 7.5% coal fly ash. Error bars indicate ± standard error of mean (n=5).

		2.5km east		2.5km west		20km west		55km west	
		Ash	No Ash	Ash	No Ash	Ash	No Ash	Ash	No Ash
Root length	*Moringa oleifera*	7.7 ± 0.47	6 ± 0.84	4.8 ± 0.49	0.8 ± 0.11	6.8 ± 0.26	1.8 ± 0.11	10.2±0.642	8.83 ± 1.122
	Moringa stenopetala	7.2 ± 0.52	6.2 ± 0.28	4.4 ± 0.37	1 ± 0.84	6.6 ± 0.39	1.5 ± 0.14	8.1 ± 0.251	7.5 ± 0.57
	Phaseolus vulgaris	10.8 ± 1.23	4.8 ± 0.71	3.9 ± 2.07	1 ± 0.38	5.6 ± 0.25	3.5 ± 0.2	13.7±0.779	12.6 ± 0.767
Shoot length	*Moringa oleifera*	8.10±0.9	5± 0.33	5.48±0.29	4.2±0.18	7.6±0.41	4.6±0.25	10.1±0.56	8.5±0.7
	Moringa stenopetala	8.30±0.84	4.7±0.64	5.0±0.62	3.5±0.39	7.83±0.55	4.2±0.27	12.2±0.86	9.8±0.68
	Phaseolus vulgaris	11.2±0.73	6.8±0.73	6.73±0.8	4.825±0.81	10.825±0.78	6.325±0.96	14.2±1.17	12.6±1.28
Root dry weight	*Moringa oleifera*	0.046±0	0.032±0	0.036±0	0.007±0	0.04±03	0.01±06	0.06±02	.0050±0
	Moringa stenopetala	0.122±0	0.046±0	0.064±0	0.016±0	0.121±0	0.025±0	0.158±0.01	0.130±0.01
	Phaseolus vulgaris	0.129±0.01	0.06±0	0.053±0.01	0.014±0	0.103±0	0.041±0	0.188±0	0.131±0
Shoot dry weight	*Moringa oleifera*	0.0953±0	0.0693±0	0.0768±0	0.0400±0	0.0870±0	0.0523±0	0.1180±0	0.0983±0
	Moringa stenopetala	0.1245±0	0.0665±0	0.0700±0	0.0478±0	0.1263±0	0.0580±0	0.1430±0	0.1290±0.01
	Phaseolus vulgaris	0.663±0.02	0.38±0.02	0.354±0.03	0.197±0.01	0.614±0.01	0.3±0.03	0.845±0.01	0.67±0.19
Root: shoot	*Moringa oleifera*	0.484±0.06	0.51±0.08	0.467±0.04	0.175±0.01	0.490±0.01	0.307±0.01	0.517±0.02	0.513±0.02
	Moringa stenopetala	0.984±0.1	0.68±0.08	0.914±0.08	0.333±0.03	0.967±0.1	0.43±0.02	1.103±0.11	1.013±0.01
	Phaseolus vulgaris	0.196±0.01	0.16±0.01	0.147±0.02	0.071±0	0.167±0.08	0.137±0.02	0.222±0.02	0.196 ±0.01

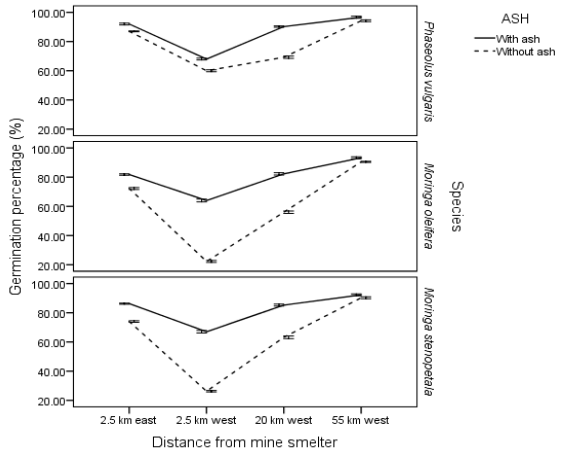

Fig. 1. Germination percentage of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris* grown in polluted soil treated with 7.5% coal fly ash. Error bars indicate ± standard error of mean (n=5).

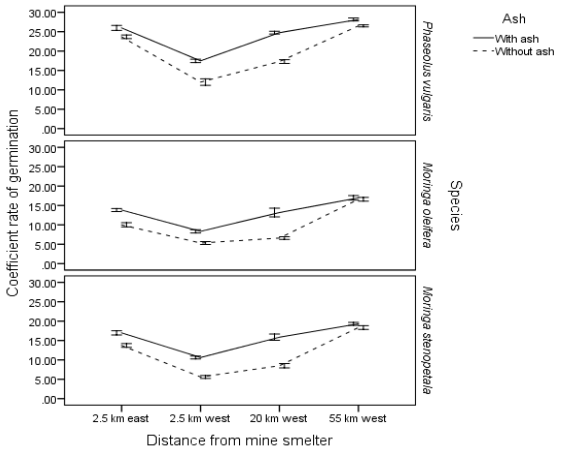

Fig. 2. Coefficient rate of germination of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris* grown in polluted soil treated with 7.5% coal fly ash. Error bars indicate ± standard error of mean (n=5).

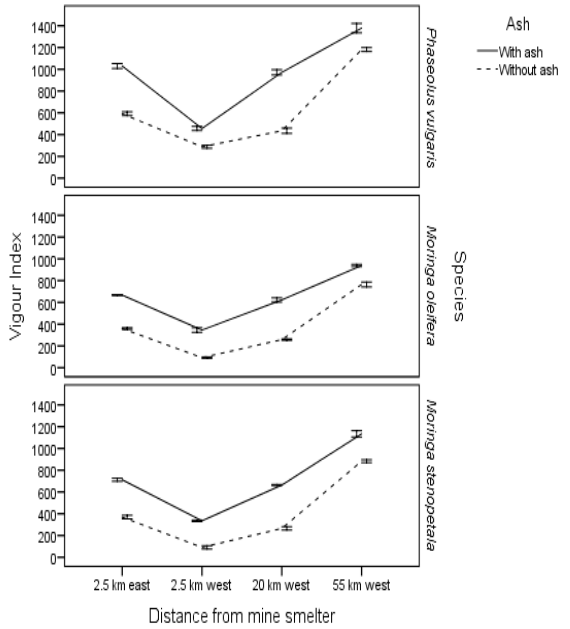

Fig. 3. Vigour index of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris* grown in polluted soil treated with 7.5% coal fly ash. Error bars indicate ± standard error of mean (n=5).

Phaseolus vulgaris, *Moringa oleifera* and *Moringa stenopetala* varied in their response to soil acidity and heavy metal stress at the four distances (sites). *Phaseolus vulgaris* performed better than both *Moringas* while *Moringa oleifera* and *Moringa stenopetala* were not significantly different ($P<0.05$) in overall performance (Fig 1- 4; Table 1). Variations observed amongst the species could possibly be attributed to their tolerance capacity and the role of genetic variation establishing individuality of species. The main initial steps in phytoremediation are the identification of species which are able to grow and develop in the contaminated/ polluted soils thus this experiment has shown that *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* have potential to be used for phytoremediation purposes since they were all able to germinate and survive in the polluted soils.

Application of 7.5% coal fly ash which had a soil pH of 10.3 (Vurayai *et al.*, 2017) enhanced all the parameters measured in this study (germination percentage, coefficient rate of germination, root and shoot growth, root and shoot dry weight, root: shoot ,vigour index and tolerance index) (Fig 1- 4; Table 1). Similar results were also observed by other scientists where application of coal fly ash resulted in higher root and shoot length of mustard (*Brassica Juncea*) (Gautam *et al.*, 2012), increased germination percentage, root and shoot length in rice and maize (Panda *et al.*, 2015).

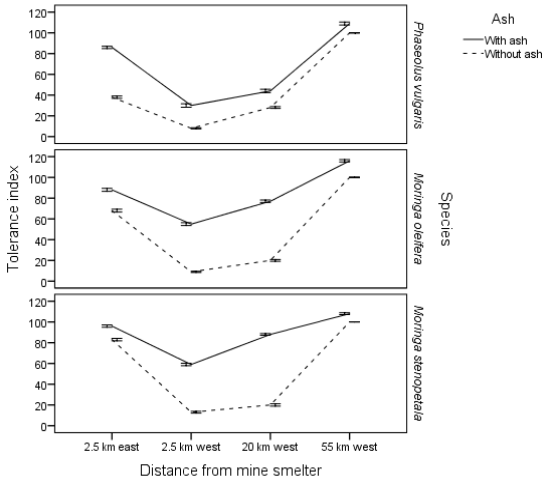

Fig. 4. Tolerance index of *Moringa oleifera, Moringa stenopetala* and *Phaseolus vulgaris* grown in polluted soil treated with 7.5% coal fly ash. Error bars indicate ± standard error of mean (n=5).

As mentioned earlier the soil pH of areas closest to the mine smelter is very low and contaminated with heavy metals. Acidic soils can be supplemented with additives having pH buffering capacity to bring their pH to neutral. Alkaline fly ash (in this case fly ash from Morupule power station, Palapye, Botswana with pH of 10.3) can therefore be used as a soil buffering agent for such problematic soils (Jala and Goyal., 2006). Coal fly ash used in this experiment increased soil pH in soil collected 2.5km west from 3.36 to 7.01, 20km west (5.63 to 7.31) 55km west (6.28 to 7.64) (control) and 2.5km east (4.3 to 7.45) (Vurayai *et al.* 2017).

The increase of soil pH in both soils can be attributed to the neutralization of H^+ by alkali salts and also due to solubilization of basic metallic oxides of fly ash in soil (Khan and Khan, 1996). At very low pH levels, the dissolution and bio-availability of plant toxic heavy metals is increased and so the incorporation of alkaline coal fly ash increases soil pH and immobilises heavy metals thus reducing their

availability to plants (Polat *et al*., 2002). This reduces heavy metal toxicity in the soil and increases overall germination and seedling growth and vigour and tolerance indexes as shown in Fig 1-4 and Table 1. While the addition of coal fly ash improves soil pH, it also simultaneously adds essential plant nutrients (Ca, Fe, Mg, K, B) to the soil (Rai *et al*., 2004; Jaya and Goyal, 2006) thus results in increased growth and in this case increased root and shoot growth which increases total dry weight (Table 1), vigour and tolerance index (Fig 3 and 4). This study therefore shows that coal fly ash has great potential for use in increasing the phytoremediation potential of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* on land east and west of the BCL Cu/ Ni mine smelter in Selibe-Phikwe, Botswana.

Conclusion

Soil acidity and heavy metal pollution reduced germination percentage, coefficient rate of germination, root and shoot growth, root and shoot dry weight, root: shoot, vigour index and tolerance index of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala*. The reduction was highest closest to the mine smelter and followed the order 2.5km west < 20km west < 2.5km east < 55km (control) and this order is correlated to soil pH and some heavy metals (Cu, Ni and Fe). *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* germinated in all soils collected east and well of the mine smelter. Their ability to germinate in polluted soil indicates tolerance to heavy metal stress and soil acidity and so they have potential for use in phytoremediation of contaminated soils around BCL Cu-Ni mine in Selebi-Phikwe. *Phaseolus vulgaris* had the highest overall germination performance than the *Moringas* but there was no significant difference between *Moringa oleifera* and *Moringa stenopetala*. Application of coal fly ash increased germination percentage, coefficient rate of germination, root and shoot growth, root and shoot dry weight, root: shoot, vigour index and tolerance index of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala*. Percentage increases followed the order 2.5km west < 20km west < 2.5km east < 55km west (control). Our results therefore suggest that coal fly ash enhances germination of *Phaseolus vulgaris*, *Moringa oleifera* and *Moringa stenopetala* in acidic and heavy metal polluted soils and can therefore be used to amend polluted soil around the mine for phytoremediation purposes.

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