USING PLANT TISSUE CULTURE TO DEVELOP PLANTS WITH ACID SOIL, HEAVY METAL TOLERANCE (AHMT), POTENTIALLY USEFUL FOR HARD-ROCK MINE LAND RECLAMATION[1]

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Abstract. Our objective for this project was to determine if we could find, collect, and tissue culture local native plants which grow directly on acid, heavy metal contaminated (AHM) soil. These plants are potentially useful for mine land reclamation and may possess acid soil, heavy metal tolerance. Explants (small actively growing plant tissue segments) were collected from fifteen plant species growing directly on degraded sites heavily impacted from past hard rock mining activities in Western Montana. These plants, when collected were rooted, surviving and growing in soils characterized by very low soil pH (about 4.0) and high concentrations of various heavy metals. Of the 15 species collected, six were successfully propagated using plant tissue culture, also known as micro-propagation. We concentrated on three of the six species and have attained commercial rates of multiplication and rooting in-vitro. These three species have been successfully acclimated and grown under commercial greenhouse conditions. Results comparing our potentially AHMT tissue cultured plants to non-selected plants of the same species (raised from seed) in test soils under greenhouse conditions will be presented. Future plans for field tests will be described and discussed.

Additional key words: Micro-cuttings, in-vitro, site-adapted

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Introduction

In Montana alone there are about 6,000 abandoned hard rock mines and milling sites; 268 of these have serious environmental and safety issues (Marty, 2000; Tafi and Neuman, 2006). Hard rock mining wastes have contaminated 3,346 miles of rivers in six Western states, including 1,118 in Montana (Clement, 2002, EPA/DOE, 2003). There are abandoned mines in 134 of the 387 National Park System units and "more than 3,200 sites with about 10,000 mine openings, piles of tailings, and hazardous structures and thousands of hectares of scarred lands" (NPS, 2008). Estimates of the cost to reclaim these lands run into the billions of dollars.

There is a human health factor associated with un-reclaimed mines. Though mine sites are often remote, there are many people, families, children and older folks living close to them. A 1997 EPA demographic study indicates that "228,145 persons and 58,996 families live within 1 mile of 306 hardrock mines" (U.S. Environmental Protection Agency, 1997). This includes 55,374 children under the age of 4. The EPA concluded that "mining and mineral processing sites do in fact have the potential to affect large numbers of people living nearby". Heavy metals in the environment cause a multitude of deleterious human health effects, including various cancers, kidney disease, and hyperthyroidism (Gillig and Kicera, 1990).

Establishing native vegetation on acid mine lands will reduce wind and water erosion and decrease contaminant leaching into sub-surface waters. Successful revegetation has been accomplished at great expense by extensive amendment of the degraded soils with lime and organic materials, or by use of cover-soils (Dollhopf et al, 1998; Jennings and Neuman, 2006, Massey, 2006, Massey and Thompson, 2006, Munshower et al, 2003, Neuman et al, 2005, Prodgers, 2003, Prodgers, 2006). However, for the most part, plant cover and species diversity remains low on many sites even though some revegetation efforts (e.g. Anaconda, Montana) began in the 1940's.

There is a need for locally-adapted native plants which are tolerant of very low soil pH and elevated heavy metal concentrations to revegetate these acid mine lands in the Rocky Mountain west (Jennings and Neuman, 2006; Neuman et al, 2005). Large numbers of acid and heavy metal tolerant (AHMT) plants are necessary because of the immense area of acid mine land that needs to be reclaimed. AHMT plants will be needed even on amended or cover-soiled sites as plant roots often extend into un-amended soil (Majerus, 2006). Also, "not all contaminated soils

are accessible or traversable with farming equipment, creating a need for seed and transplants of AHMT plant material" (Deer Lodge Conservation District et al, 2004).

Some researchers have experimented with using native plant seed collected from such sites as a way to produce AHMT locally adapted native plants (EPA/DOE, 2004, Majerus, 2006, Marty, 2000). Though more research needs to be done, it appears that plants from this seed do better on degraded sites than open source seed of the same variety (Majerus, 2006, Majerus and Majerus, 2008). However, many desirable native plants are difficult to propagate by seed for a variety of reasons such as poor germination, poor seed production, short seed viability, and complex dormancy (Dorner, 2006, Munshower, 1995, Munshower, 1998, Olson and Zabinski, 2003, Walker and Shaw, 2005). For some desirable native plants it may be difficult or impossible to collect sufficient quantities of local seed for seed increase or direct seeding. Vegetative production (e.g. by cuttings) also is not successful for many species. Plant tissue culture is currently being used to produce plants for reclamation in Australia and has been investigated for phosphate mine reclamation in Florida (Deghan et al, 1989, Kalmbacher et al, 2004, Norcini et al, 2003, Willyams, 2005, Willyams, 2005b).

AHMT locally adapted tissue cultured plants could be used for seed increase or transplantation onto degraded sites. Producing plants via tissue culture has several advantages:

- Plants are genetically identical, i.e. clones, and will have the same characteristics, e.g. AHMT, as the parent material.
- They can be very site-specific, propagated from parent plants present on the exact location, if desired.
- Multiplication can be rapid, 4 to 8X every 4 to 6 weeks,
- Plants may be quicker to mature, flower and set seed,
- Many thousands of plants can be cultured in a very small space.

No commercial plant nurseries or tissue culture labs provide any AHMT locally adapted tissue cultured plants, and few produce AHMT plants of any sort. The ARS-USDA Bridger Plant Materials Center does have seed from AHMT plants which is currently being increased (Majerus, 2003, Majerus, 2008).

Materials and Methods

Collection Sites, Plants Collected and Collection Methods

SMK Plants personnel made 4 trips to Southwestern Montana to assess potential sites, document site soil characteristics and collect explants. Explants are small, actively growing plant pieces, usually meristems, internodes, root tips or seeds. The source plant is not destroyed by this sampling. The first two trips we were accompanied by Reclamation Research Group LLC (RRG) personnel. We targeted and collected 15 different plant species from 6 different sites (Table 1). We took soil samples from each individual collection site and marked each site by GPS coordinates and physical markers.

To help ensure that the plants from which the explants are taken were likely AHMT, soil samples were taken from the root zone of the plants. We carefully excavated around the root zone of the plants and made careful observations as to whether roots, rhizomes, and root hairs were visible growing in the contaminated soil, and documented this photographically.

Collection Methods. Explants were excised from the parent plants carefully with sharp mini-pruners. We tested three different collection methods. The collection methods were: 1) place plant material (explants) in moistened towel in poly bag; 2) cut sections into a collection solution (all solutions used for collecting, initiation, multiplying and rooting are not detailed and are proprietary) contained in small glass vials; 3) rinse cut sections with Clorox solution and sterile water and then put in collection solution.

Plant Tissue Culture

Culture Initiation. Once in the lab, stem sections, whole seed pods/fruit, or seeds were sterilized in a standard Clorox solution, placed on initiation medium (proprietary), and cultured for up to 8 weeks under fluorescent lights at room temperature. Data was taken at 4 and 8 weeks on color, new growth, death, and contamination (i.e. bacterial and/or fungal growth). Explants were considered dead if they were discolored, showed no sign of new growth, and the interior tissue was brown. After the initial initiation trials from the first two collections, 7 species were selected for repeat collection and further study.

Table 1. Plant Species and Locations

Plant Species	Scientific Name	Plant Type	Location
Bastard Toadflax	*Comandra umbellata*	forb	Keating West
Fringed Sage	*Artemisia frigida*	sub-shrub	Keating West
Woods Rose	*Rosa woodsii*	sub-shrub	Keating Test Plots
Sandbar Willow	*Salix exigua*	shrub	Silver Bow Creek
Saltgrass	*Distichlis spicata*	grass	Silver Bow Creek
Creeping Juniper	*Juniperus horizontalis*	shrub	Opportunity Ponds
Narrowleaf Cottonwood	*Populus angustifolia*	tree	Keating Test Plots
Evening Primrose	*Oenothera cespitosa*	forb	Keating North
American Vetch	*Vicia americana*	forb	Keating North
Water Birch	*Betula occidentalis*	shrub	Clark Fork
Sandbar Willow	*Salix exigua*	shrub	Clark Fork
Grey Horsebrush	*Tetradymia canescens*	shrub	Anaconda
Penstemon sp	*Penstemon eriantherus*	forb	Anaconda
Oxytropis sp	*Oxytropis*	forb	Anaconda
Alum Root	*Heuchera parvifolia*	forb	NW Energy Substation
Chokecherry	*Prunus virginiana*	shrub	Mt Haggin

Multiplication. Multiplication trials were conducted on *Heuchera parvifolia*, *Vicia americana*, and *Oenothera cespitosa*. For each species the multiplication trial involved placing shoot tips, nodal segments, or whole seedlings in test tubes with multiplication media (proprietary) in a factorial design. We tested 3 or in some cases 4 levels of N6-benzyladenine (BA), and 2 levels of naphthalene acetic acid (NAA) or Murashige and Skoog salts (MS) concentrations. These tubes were cultured under standard production conditions (see above under Culture Initiation) for

4 weeks, after which a shoot from each treatment was transferred to fresh treatment medium and cultured another 4 weeks (total of 8 weeks). Data was collected on color, general health (numeric scale), and number and length of new shoots at 2 and 4 weeks and an optimum multiplication media was chosen for each species.

Rooting. Rooting trials were then done for *Vicia americana*, *Oenothera cespitosa*, and are currently being run for *Heuchera parvifolia*, using material growing on the optimum multiplication media (proprietary). The procedure was similar to the multiplication trial except the rooting media had varying levels of indole-3- butyric acid (IBA) and MS salts. At 6 weeks, data was collected similar to multiplication trials and included number of roots and length of longest root.

Results and Discussion

Plants Collected

The plants collected (Table 1) included tree species, shrubs, perennial forbs and one grass. These particular plant species were chosen and collected because each could serve very useful purposes in reclaiming mine land:

- Structure for animal habitat – the trees and shrubs (e.g. *Prunus virginiana*)
- Nitrogen fixation – *Vicia americana* and *Oxytropis sp*.
- Vegetative cover – all, but especially *Distichlis spicata*, *Salix exigua*, and *Juniperus horizontalis*
- Streambank stabilization – *Salix exigua, Populus angustifolia,* and *Betula occidentalis*
- Native species diversity – i.e. *Comandra umbellata*, *Heuchera parvifolia*, and *Oenothera cespitosa*.
- Animal browse – especially *Rosa woodsii* and *Prunus virginiana*
- Erosion control – especially *Salix exigua* and *Juniperus horizontalis*.

The pictures in Fig. 1 and Fig. 2 show that the plants' root systems extended into the contaminated soils. Visual observations revealed small roots and root hairs surviving there. Figure 3 shows that the soil surface was around pH 4.5. After determining which plants had good tissue culture potential (based on initiation trials), the stored root zone soil samples were analyzed for heavy metal concentrations, soil pH, and soil characteristics (Energy Labs, Billings, MT, Table 2).

Figure 1. *Oenothera cespitosa*, roots and rhizomes in tailings.

Figure 2. *Distichlis spicata* var. stricta, rhizomes in fluvial mining waste deposits.

Figure 3. *Oenothera cespitosa*, low pH revealed on quick field soil test.

Soil samples from the root zone indicated that the plants were indeed growing in acidic and/or high metal availability soils (Table 2). For *Oenothera cespitosa*, seedlings were observed healthy and growing in the contaminated soil (Fig. 3). All plant material arrived at the lab turgid, with normal color. Cold storage of the plant material for longer periods than 3 weeks resulted in browning and visible fungal growth on some plant material and therefore plants should be initiated into culture as soon as possible to avoid this.

Table 2: AHMT Sites: Site Location and Soil Properties.

					Total Metal Content , mg/kg, Sampling Depth 0-6 inches								
Collection Site	**Screning Soil pH***	**Laboratory Soil pH**	**EC (mmhos/cm)**	**Organic Matter (%)**	**Al**	**As**	**Cd**	**Cu**	**Pb**	**Mn**	**Mo**	**Ni**	**Zn**
Keating West	3.0												
Keating West	5.3												
Keating Test Plots	3.7												
Silver Bow Creek	5.2	4.7	0.46	3.2	18300	510	7	1530	1050	1510	12	9	2260
Silver Bow Creek	4.0	4.1	9.95	1.4	10800	356	11	1500	737	1230	9	7	3460
Opportunity Ponds	6.2												
Keating Test Plots	4.3												
Keating North	4.4	4.3	4.68	0.4	10200	135	ND	58	58	291	ND	ND	288
Keating North	5.4	5.9	2.51	0.9	6890	115	ND	232	62	458	ND	ND	352
Clark Fork FWP	6.0												
Anaconda, State land	4.9												
Anaconda, State land	7.0												
Anaconda, State land	6.2												
NW Energy Substation	5.2	5	0.35	3.3	15700	590	11	976	204	616	ND	12	582
Mt Haggin	5.1												

* Soil pH is the screening level soil pH measured using a 1:1 slurry of soil and deionized water

& Sent to Energy Labs August 10, 2007 for determination of pH, EC, OM, total Al, As, Cu, Pb, Mn, Mo, Zn, Ni, Cd

ND=Not Detected

Collection Methods

The collection methods were: 1) place plant material (explants) in moistened towel in poly bag; 2) cut sections into a collection solution contained in small glass vials; 3) rinse cut sections with Clorox solution and sterile water and then put in collection solution. In general (Table 3), explants survived better when collected directly into a collection solution (Methods 2, 3, or 4) than simply bagged (<20%, Method 1).

Table 3: Initiation Trials: Contamination and Survival by Collection Method

METHOD	TOTAL # ATTEMPTED	% CONTAMINATED	% SURVIVAL
#1- bagged with moistened towel	616	18	18
#2 – submerged in collection solution	192	9	51
#3 – 10 second Clorox solution soak, then #2	92	11	78
#4 - #3 without sterilization at initiation	33	6	55

Treatment of excised plant material at field collection site and prior to initiation in the laboratory. All species, explant types, and collection dates combined.

Culture Initiation

Many of the 15 species collected in May did not survive the initiation process (Table 4). This was unexpected since young, actively growing tissue is often used for tissue culture initiation. Of particular note is that some species (e.g. *Heuchera parvifolia*) for which tissue culture protocols had been devised and in use (SMK Plants, proprietary), did poorly (0% survival of young shoots) when collected from AHM sites. These results emphasize the need to treat each species growing on AHM sites as new to the tissue culture process, regardless of the previous work done for that species. Contamination (i.e. bacterial and fungal growth) and survival was low for most cultures, indicating the sterilization method disinfested the explants but may have also damaged the explants (Table 4).

In general (all other factors combined), survival was better for:

- explants collected directly into a collection solution (>50%) than simply bagged (<20%, Table 3).
- plants collected in July (54%) than in May (<10%, Table 5);
- seeds (59%) than shoots (11%, Table 6);

Using these factors (later collection date, seed collection, collecting into collection solution) will improve the initiation success.

Of the six species (Table 4) that had sufficient initiation success (i.e. survival rates equal to or better than 19%) to maintain the cultures, we chose three forbs (*Heuchera parvifolia*, *Vicia americana*, and *Oenothera cespitosa*) initiated from seeds to continue research with. Success on the remaining three will depend on further culturing and initiation of more plant material.

Table 4: Initiation Trials: Contamination and Survival by Plant Species

SPECIES	COLLECTION DATES	TOTAL # ATTEMPTED	% CONTAMINATED	% SURVIVAL
Artemisia frigida	5/15	35	34	0
Betula occidentalis	5/24	58	10	0
Comandra umbellata	5/15	47	9	9
Distichlis spicata	5/15, 7/01, 8/17	149	26	42
Heuchera parvifolia	5/24 & 7/01	182	7	46
Juniperus horizontalis	5/15 & 8/17	127	19	19
Oenothera cespitosa	5/24 & 7/01	185	5	21
Oxytropis sp.	5/24	37	11	3
Penstemon eriantherus	5/24	31	3	6
Populus angustifolia	5/24	23	0	0
Prunus virginiana	5/24 & 8/13	47	0	0
Rosa woodsii	5/15	34	18	3
Salix exigua	5/15 & 7/01	162	27	20
Tetradymia canescens	5/24	52	0	2
Vicia americana	5/24 & 7/01	128	9	43

All plants collected in 2007. All explant types, collection dates, and collection methods combined.

Table 5: Initiation Trials: Contamination and Survival by Collection Date

COLLECTION DATE	TOTAL # ATTEMPTED	% CONTAMINATED	% SURVIVAL
05/15/07	434	25	9
05/24/07	538	4	4
07/01/07	457	10	54

Date of field collection all species, explant types, and collection methods combined.

Table 6: Initiation Trials: Contamination and Survival by Explant Type

EXPLANT TYPE	TOTAL # ATTEMPTED	% CONTAMINATED	% SURVIVAL
Shoots	1115	15	11
Seeds	314	2	59

All species, collection dates, and collection methods combined. Seeds are both mature and immature.

Multiplication

All three species, *Vicia americana*, *Oenothera cespitosa*, and *Heuchera parvifolia* produced new shoots after 4 weeks of culture and maintained shoot production after subculture (Fig. 4). Due to high variability within treatments and low replicate numbers (due to amount of material available) many treatments were not significantly different in the average number of shoots or nodes produced. However, the highest multiplication rates achieved (4x to 15x, Table 7) were at or above the 4x rate of tissue cultured plants produced by SMK Plants for ornamental nursery production. These multiplication rates mean that our AHMT plants will be economical to produce and competitive with plants produced by other means, i.e. seed or vegetatively.

Table 7: Treatment with Highest Multiplication Rate for 3 Species

SPECIES	TOTAL # SHOOTS	AVERAGE #/EXPLANT	SD
Heuchera parvifolia	19	3.8 shoots	±2.8
Oenothera cespitosa	77	15.4 shoots	±9.6
Vicia americana	21	4.2 nodes	±2.3

Total # of new shoots is for 5 explants (total tested per treatment.)

Rooting

In vitro rooting was successful (up to 100%, Table 8) for both *Vicia americana* and *Oenothera cespitosa*. Optimum treatments for rooting were single shoots on a medium with IBA (indole-3- butyric acid) for *Oenothera cespitosa* and shoot clumps on a medium without IBA for *Vicia americana*. Figure 5 shows outstanding in vitro rooting of a leguminous species, *Vicia americana*. Though not always necessary, rooting *in vitro* is beneficial for improving the survival of the plants when transferred to greenhouse conditions. Preliminary indications are that

Heuchera parvifolia will also readily root *in vitro*. This success in rooting means that our plants will be economical to produce and competitive with plants produced by other means.

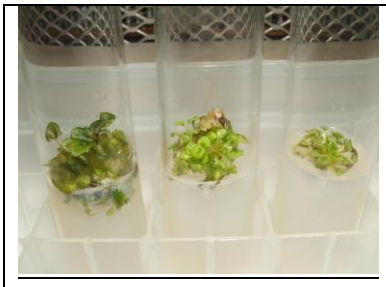
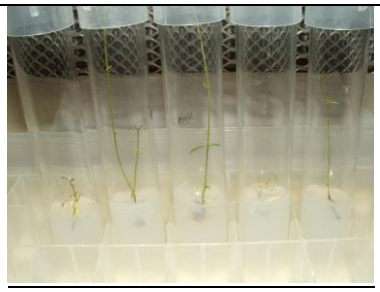
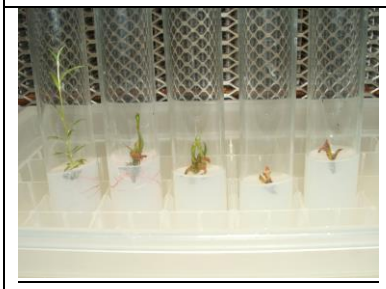
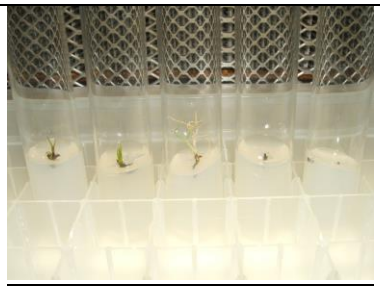
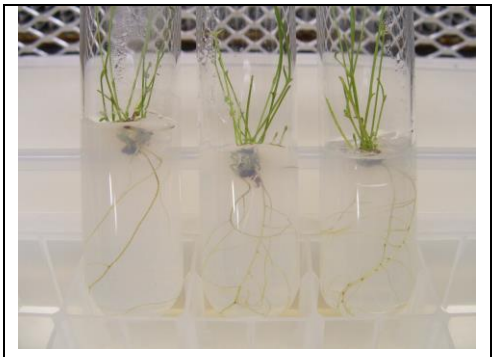
		
Heuchera parvifolia (multiplication experiment)	***Oenothera cespitosa*** (multiplication experiment)	***Vicia americana*** (multiplication experiment)
		
Salix exigua	***Distichlis spicata* var. stricta**	***Juniperus horizontalis***

Figure 4. Plants in Culture



Vicia americana rooting in flasks

Figure 5. Rooting Tissue Cultured Plants

Table 8: Treatment with Highest Rooting Rate for 3 Species

SPECIES	% ROOTED	AVERAGE # OF ROOTS	SD
Oenothera cespitosa	100	9.0	2.8
Vicia americana	100	1.3	0.5
Heuchera parvifolia	100	8.4	3.2

Production

The goal was to produce 1000 tissue cultured plants for each species within seven months of collection, and determine if cost of production would be similar to other tissue cultured nursery plants. All cultures for the successful species were routinely transferred onto multiplication media until 1,000 new shoots were produced. This was easily accomplished well within seven months for *Heuchera parvifolia*, *Vicia americana*, and *Oenothera cespitosa* (Table 9). Three species of importance, *Distichlis stricta*, *Salix exigua*, and *Juniperus horizontalis*, did not have sufficient initial cultures for the multiplication and rooting trials, but will continued to be cultured and further studied in Phase II. The multiplication rates and rooting capacity of the three species are as good as or better than many commercially tissue cultured plants and therefore the cost would be similar.

Table 9: Time to Produce 1,000 Shoots of 3 Species

SPECIES	COLLECTION DATE	INITIATATION DATE	# INITIATED	1000 SHOOTS DATE	TOTAL TIME (MONTHS)
Heuchera parvifolia	07/01/07	07/19/07	50	12/06/07	5
Oenothera cespitosa	07/01/07	07/20/07	55	10/30/07	3
Vicia americana	07/01/07	07/25/07	50	10/25/07	3

Conclusions and Future Plans

We proved the principle that we can find, collect, and tissue culture local native plants which grow directly on acid, heavy metal contaminated (AHM) soil. We discovered how to use plant tissue culture to get these plants to multiply and root *in vitro* in the same way as commercial ornamental plant species. Production costs of these plants will be similar to commercial ornamental plants and were estimated to be economical for reclamation.

Of the 15 plant species collected, 4 are currently growing in clean culture. These are *Heuchera parvifolia*, *Vicia americana*, *Oenothera cespitosa,* and *Distichlis spicata.* These four have survived transfer to commercial greenhouse conditions and have acclimated to greenhouse conditions in pots. *Heuchera parvifolia*, *Vicia americana*, and *Oenothera cespitosa* were outplanted on contaminated soils at our Keating tailings site in fall of 2008.

We have recently concluded a greenhouse test comparing our tissue cultured, local site-adapted plants to seedlings of the same species but whose seed did not come from AHM contaminated sites (non-adapted). Each type of plant was potted in two low pH, heavy metal contaminated test soils and in potting soil. Productivity measurements were taken at intervals for all plants. These results are still being analyzed.

We are also comparing our tissue cultured, site-adapted plants to non-local, non-adapted seedlings in a field test at our Keating tailings site. Data collection comparing our tissue cultured plants to their seedling counterparts will be collected in the spring of 2009 and in following years to prove that our site-selected plants have advantages. Approximately 1 month after out-planting, all plants seemed to be surviving (visual observation).

We have had some significant disappointments. We were not successful in getting 9 of the plant species we collected into clean culture. These failures include such desirable species as water birch, fringed sagewort, narrowleaf cottonwood, *Oxytropis sp*., and grey horsebrush. Still, we have more species in culture than we expected in our research proposal. These include key ecosystem components such as a nitrogen-fixer (*Vicia Americana*) and stress tolerant, hardy grass species (saltgrass, *Distichlis spicata*).

The goal for our future research is to test our tissue cultured plants for AHM tolerance in both greenhouse and field tests. We will also tissue culture other species of locally adapted native plant species growing on AHM soils, then successfully grow and overwinter them under

commercial green house conditions, and eventually commercialize them for revegetating AHM contaminated sites.

Revegetation of degraded mine land will benefit society by reducing wind and water erosion from these lands, reducing human exposure and improving human health and living conditions. Reclamation and revegetation will also improve wildlife habitat, improve aesthetics and recreational values, and may help endangered species.

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