

CAROLINA RAMOS MONTAÑO

**TOLERÂNCIA DE *Alnus acuminata* (ALISO) AO ARSÊNIO,
DURANTE A GERMINAÇÃO E O DESENVOLVIMENTO INICIAL**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2018

**Ficha catalográfica preparada pela Biblioteca Central da Universidade
Federal de Viçosa - Câmpus Viçosa**

T

R175t
2018
Ramos Montaña, Corolina, 1977-
Tolerância de *Alnus acuminata* (Aliso) ao arsênio, durante a
germinação e o desenvolvimento inicial / Corolina Ramos
Montaña. – Viçosa, MG, 2018.
xi,70 f. : il. (algumas color.) ; 29 cm.

Inclui apêndices.

Orientador: Juraci Alves de Oliveira.

Tese (doutorado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. *Alnus acuminata* - Desenvolvimento - Efeito dos metais
pesados. 2. Germinação. 3. Solo - Teor de arsênio. 4. Carvão -
Minas e mineração. I. Universidade Federal de Viçosa.
Departamento de Biologia Geral. Programa de Pós-Graduação
em Fisiologia Vegetal. II. Título.

CDD 22. ed. 583.74

CAROLINA RAMOS MONTAÑO

**TOLERÂNCIA DE *Alnus acuminata* (ALISO) AO ARSÊNIO,
DURANTE A GERMINAÇÃO E O DESENVOLVIMENTO INICIAL**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae*.

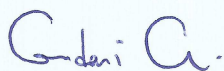
APROVADA: 20 de fevereiro de 2018.



Eduardo Euclides de Lima e Borges



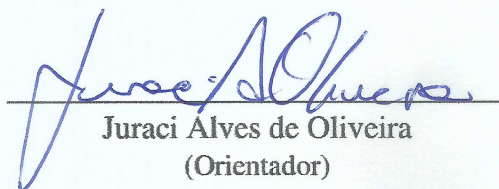
Dimas Mendes Ribeiro



Jorge Alberto Condori Apfata



Eduardo Fontes Araujo



Juraci Alves de Oliveira
(Orientador)

AGRADECIMIENTOS

Agradezco a la Universidad Federal de Viçosa por abrir para mí las puertas del conocimiento y la investigación de máximo nivel, y a la Universidad Pedagógica y Tecnológica de Colombia por avalar mi comisión de estudios doctorales.

A los profesores de la Posgraduación: Fábio da Matta, Adriano Nunes Nesi, Eduardo Fontes, Dimas Ribeiro, Herminia Martinez, Eduardo Borges, Samuel Vitor Martins, y especialmente a mi orientador Juraci Alves de Oliveira por su valioso acompañamiento a lo largo de estos cuatro años de doctorado.

A Todos mis colegas del laboratorio de Biofísica, con quienes compartimos ideas y pusimos a prueba metodologías intentando mejorar la calidad de la pesquisa, y a Taline Gonçalves por su colaboración durante el desarrollo del proyecto.

A mi familia: Ceci, Oscar, Miguelito, eternos entusiastas de mis aventuras y pequeñas luces que en la lejanía me muestran el camino de regreso. A mi tío Salvador por su profunda confianza.

A esos grandes amigos de Tunja que desde Colombia me han brindado toda la ayuda que he necesitado para culminar este doctorado exitosamente; Adriana, Maribel, Gabriel, Mayer, Mechis, Jairo y Zaida.

A mis grandes amigos acá en Brasil, almas colombianas, mexicanas, brasileras, ecuatorianas, peruanas, costarricenses, mozambiqueñas que pusieron su cariño compartiendo inolvidables momentos conmigo y mantuvieron mi ánimo y energía.

RESUMO

RAMOS-MONTAÑO, Carolina, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Tolerância de *Alnus acuminata* (Aliso) ao arsênio, durante a germinação e o desenvolvimento inicial.** Orientador: Juraci Alves de Oliveira.

Na Colômbia, a mineração de carvão é uma das atividades econômicas mais frequentes, especialmente na região Andina do estado de Boyacá. Até o momento não se tem conhecimento de estudos envolvendo a determinação das concentrações de metais pesados ou metaloides no solo, e o governo obriga às empresas de mineração a fazer reflorestação nos solos pós-mina, sem que existam espécies recomendadas para isso. Esta pesquisa abordou a problemática da mineração de carvão com dois objetivos principais: (1) Estabelecer se a mineração de carvão está associada a altas quantidades de metais pesados ou do arsênio, e se encontrar alguma espécie de árvore que esteja distribuída em lugares com altas concentrações de esses elementos traça e (2) aprofundar no estudo germinativo de essa espécie para determinar se tem algum tipo de tolerância e possa ser usada em plenos de reflorestação de solos pós-mineração. Num primer estudo na municipalidade de Samacá (Boyacá-Colômbia), foram traçadas linhas de amostragem de 50 m desde os resíduos de carvão até a zona mais conservada, para observar as mudanças na composição e diversidade ao longo da distância. Em cada um de quatro pontos de amostragem foram determinadas as concentrações de alumínio, arsênio, cobre, ferro, manganês, níquel, chumbo e zinco. Os resultados mostraram que, exceto do arsênio, nenhum dos elementos traça analisados estão presentes em concentrações de risco toxicológico, mas a diversidade muda consideravelmente desde a fonte de carvão ao exterior, com algumas espécies que seriam intolerantes por estarem presentes somente na zona mais distante. A única espécie de árvore encontrada na zona mais próxima aos rejeitos de carvão foi *Alnus acuminata*, espécie neotropical de nome comum aliso. Num segundo estudo, as características germinativas de *A. acuminata* foram avaliadas para estabelecer quais eram as melhores condições que maximizam a germinação. Os resultados demonstraram que as sementes de *A. acuminata* podem ser conservadas durante meses a temperaturas menores a 5°C; que um pre-tratamento de estratificação em frio por 48 horas melhora a germinação da espécie sempre que essas sementes germinem em presença de luz e que as condições recomendadas para incubação são: Temperaturas alternas 20-30°C com 12 horas de fotoperíodo,

semeadura em papel germitest. Finalmente, um terceiro estudo determinou o efeito do arsênio na germinação e desenvolvimento inicial de *A. acuminata* com e sem pretratamento germinativo de estratificação. Os resultados demonstraram que essa estratificação, aplicada como uma pre-embebição a 3°C por 48 horas aumenta a germinação em 50% e melhora o desenvolvimento inicial, acelerando a emergência da primeira folha verdadeira. Este efeito faz que inclusive as sementes germinadas que vem de tratamentos com arsênio tenham um crescimento ótimo sem diferenças significativas com os controles até a idade de 70 dias. Com a altura de essas plântulas foi calculado o índice de vigor das sementes, que basicamente foi incrementado junto com a tolerância ao arsênio em mais de 50% em comparação com as sementes que não tinham estratificação. A conclusão geral de esta pesquisa de doutorado é que *A. acuminata* apresenta tolerância germinativa ao arsênio, sempre que as suas sementes recebam um pre-tratamento de embebição em frio, e que esta descoberta deve ser usada para avaliar a implementação de *A. acuminata* em planos de recuperação de solos afetados pela mineração de carvão em Colômbia.

ABSTRACT

RAMOS-MONTAÑO, Carolina, D.Sc., Universidade Federal de Viçosa, February, 2018. **Tolerance to arsenic in *Alnus acuminata* (Aliso), during the germination and early development.** Adviser: Juraci Alves de Oliveira.

In Colombia, coal mining has become one of the most frequent economic activities, especially in the Andean region in the department of Boyacá. Currently it is unknown if there is an increase in the concentrations of heavy metals and metalloids in the soil, and the owners of the mines are requested by the government to reforest the post-mining soils, without any species specifically recommended for it. The present investigation addressed the problem of coal mining with two main objectives: (1) To establish if coal mining is associated with high amounts of heavy metals or metalloid arsenic, and find some tree species that was distributed in the proximity of the coal tailings where trace elements are concentrated and (2) To deep in the study of that species to determine if it has any germination tolerance and if it has potential to be used in reforestation plans in post-mining soils. In a first study carried out in the municipality of Samacá (Boyacá-Colombia), transects of 50 m were assembled from the mining tailings to the most conserved area of vegetation, to observe how the diversity and composition changes along the distance. In each of four sampling points, the concentration of aluminum, arsenic, copper, iron, manganese, nickel, lead and zinc were determined. As a result, it was found that, with the exception of arsenic, none of the trace analyzed elements are found in concentrations of toxicological risk, but the diversity changes considerably from the coal source to the exterior; some species would be considered as intolerant because they are present only in the most distant zone. The only tree species found in the closest zone to the coal wastes was *Alnus acuminata*, a species native from the Neotropic and commonly known as alder. In a second study, the germination characteristics of *A. acuminata* were evaluated to establish the best conditions that maximize its germination. The results showed that seeds of *A. acuminata* can be conserved for months at temperatures below 5°C. A pretreatment of cold stratification during 48 hours improves the germination in presence of light and the best conditions for germination were sowing on germitest paper, alternate temperatures 20-30 °C with 12 hours of photoperiod. Finally, in a third study, the effect of arsenic on the germination and early development of *A. acuminata* with and without a germination

pretreatment of stratification was determined. The results of this last study showed that stratification, applied as a cold preimbibition at 3 °C for 48 hours, increases germination by 50% and improves early development, accelerating the emission of the first true leaf. This effect means that even the germinants that come from arsenic treatments have an optimal growth without significant differences with the controls, up to the age of 70 days. With the height of the seedlings was calculated the vigor index of the seeds, which was basically increased in more than 50% in relation to the seeds that had not had a cold pretreatment. Similar results were found with the index of tolerance to arsenic. The general conclusion of this doctoral research is that *A. acuminata* presents germinative tolerance to arsenic, provided that its seeds receive a pre-treatment of cold imbition, and this discovery should be used to evaluate the implementation of *A. acuminata* in plans for recovering of soils affected by coal mining in Colombia.

RESUMEN

RAMOS-MONTAÑO, Carolina, D.Sc., Universidade Federal de Viçosa, febrero de 2018. **Tolerancia de *Alnus acuminata* (Aliso) al arsénico, durante la germinación y el desarrollo temprano.** Orientador: Juraci Alves de Oliveira.

En Colombia, la minería de carbón se ha convertido en uno de las actividades económicas más frecuentes, especialmente en la región Andina en el departamento de Boyacá. No existe hasta la fecha un estudio que determine si existe un aumento en las concentraciones de metales pesados y metaloides en el suelo, y los dueños de las minas son obligados por el gobierno a hacer reforestación de los suelos post-minería, sin que haya especies recomendadas para ello. La presente investigación abordó la problemática de la minería de carbón con dos objetivos principales: (1) Establecer si la minería de carbón está asociada a altas cantidades de metales pesados o del metaloide arsénico, y encontrar alguna especie arbórea que se distribuya en lugares donde se concentren esos elementos traza y (2) Profundizar en el estudio germinativo de dicha especie para determinar si efectivamente tiene algún tipo de tolerancia y tiene potencial para ser usada en planes de reforestación en suelos post-minería. En un primer estudio realizado en el municipio de Samacá (Boyacá-Colombia), fueron montados transectos de 50 m desde los residuos de minería hacia la zona más conservada de vegetación, para observar cómo cambia la diversidad y composición a lo largo del transecto. En cada uno de cuatro puntos de muestreo fueron determinadas las cantidades de aluminio, arsénico, cobre, hierro, manganeso, níquel, plomo y zinc. Como resultado se encontró que, a excepción del arsénico, ninguno de los elementos traza analizados se encuentra en concentraciones de riesgo toxicológico, pero la diversidad cambia considerablemente desde la fuente de carbón hacia el exterior, con algunas especies que definitivamente serían intolerantes por estar presentes únicamente en la sección más distante. La única especie arbórea encontrada en la sección más próxima a los desechos de carbón fue *Alnus acuminata*, especie nativa del Neotrópico y conocida comúnmente como aliso. En un segundo estudio, las características germinativas de *A. acuminata* fueron evaluadas para establecer cuáles eran las mejores condiciones que maximizaban su germinación. Los resultados demostraron que las semillas de *A. acuminata* pueden ser conservadas durante meses a temperaturas menores de 5°C, que un pretratamiento de estratificación en frío durante 48 horas mejora la germinación de la especie siempre

que las semillas germinen en presencia de luz y que las mejores condiciones para la incubación fueron temperaturas alternas 20-30°C con 12 horas de fotoperiodo, con siembra en papel germitest. Finalmente, en un tercer estudio se determinó el efecto del arsénico en la germinación y desarrollo temprano de *A. acuminata* con y sin pretratamiento germinativo de estratificación. Los resultados de este último estudio demostraron que esa estratificación, aplicada como una preimbibición en frío a 3°C durante 48 horas, aumenta en un 50% la germinación y mejora el desarrollo temprano, acelerando la emisión de la primera hoja verdadera. Ese efecto hace que incluso los germinados que provienen de tratamientos con arsénico tengan un crecimiento óptimo sin diferencias significativas con los controles, hasta la edad de 70 días. Con la altura de las plántulas en esa edad se calculó el índice de vigor de las semillas, que básicamente fue aumentado junto con la tolerancia al arsénico en más de un 50% con relación a las semillas que no había tenido un pretratamiento en frío. La conclusión general de esta investigación de doctorado es que *A. acuminata* presenta tolerancia germinativa al arsénico, siempre que sus semillas reciban un pretratamiento de imbibición en frío, y que este descubrimiento debe ser usado para evaluar la implementación de *A. acuminata* en planes de recuperación de suelos afectados por minería de carbón en Colombia.

SUMÁRIO

A) INTRODUÇÃO GERAL	1
B) CAPÍTULOS:	
Capítulo 1: ARSENIC AND HEAVY METALS IN SOILS AFFECTED BY COAL MINING IN SAMACÁ (COLOMBIA)	2
Abstract.....	2
Introduction.....	3
Methods	4
Study Area	4
Sampling and analysis of Soils.....	5
Specific extractions for As	6
Vegetation sampling and analysis of biodiversity.....	6
Statistical analysis	6
Results	7
Figure 1. Exchangeable and organic content of heavy metals and the metalloid As in soils of four sites affected by coal mining.	8
Figure 2. Speciation of As in soils of four sites affected by coal mining.	9
Table 1. Physical and chemical properties of soils in the study area and associations with total or exchangeable concentrations of trace elements.	9
Table 2. Plant species and corresponding abundance in four sites influenced by coal mining in Samacá (Colombia).....	11
Figure 3. Changes in plant diversity from the coal residuals of mines toward 50 m of distance, in four sites of Samacá (Colombia).	13
Figure 4. Cluster analysis of vegetation in the proximity of coal mine residuals.	14
Discussion.....	15
Conclusions.....	17
References.....	17
Appendices.....	22
 Capítulo 2: EFECTO DE DIVERSOS FACTORES EN LA GERMINACIÓN Y DESARROLLO TEMPRANO DE <i>Alnus acuminata</i> Kunt (Betulaceae)	 24

Resumen	24
Introducción	25
Metodología	27
Procedimiento base	27
Experimento 1	27
Experimentos 2-6	28
Análisis estadístico	29
Resultados	30
Figura 1. Germinación de <i>A. acuminata</i> en tres cosechas diferentes, bajo condiciones estándar de 25°C y fotoperíodo 12/12 h.	30
Figura 2. Efecto de la temperatura y el sustrato en semillas de <i>A. acuminata</i> de cosecha 2014 y 2015, en condiciones de oscuridad.	31
Figura 3. Efecto de la edad de diferentes semillas (tiempo de almacenamiento) en la germinación de <i>A. acuminata</i>	32
Figura 4. Efecto de estratificación húmeda en frío (4°C) de 7, 15, 30, 45 y 60 días, en la germinación de <i>A. acuminata</i>	33
Figura 5. Efecto del pretratamiento de estratificación húmeda a 4°C versus preimbibición a 25°C, en la germinación de semillas con y sin fotoperíodo de 12/12 horas.	35
Figura 6. Efecto de la luz durante la germinación, en el desarrollo hasta plántula fotosintética con 2 o más hojas verdaderas, a lo largo de 35 días de seguimiento en fotoperíodo 12/12 h.	35
Discusión	34
Conclusiones	38
Referencias	38
Apéndices	41
Capítulo 3: MOIST PRE-CHILLING IMPROVES THE GERMINATION TOLERANCE OF THE ANDEAN TREE <i>Alnus acuminata</i> TO ARSENIC	44
Abstract	44
Introduction	45
Materials and Methods	47
Chilling moist pretreatment, Leaking and Germination	47

Early development of germinants	47
Determination of arsenic in germinants	48
Biochemical analysis.....	48
Antioxidant activity.....	49
Table 1. Composition of solutions for enzymatic antioxidative reaction.....	50
Vigor and Tolerance.....	50
Statistical analysis	51
Results	51
Figure 1. Effect of stratification and arsenic on germination of <i>Alnus acuminata</i>	52
Figure 2. Early development of <i>A. acuminata</i> under 0, 2.5, 5.0, 7.5 and 10 mg L ⁻¹ of As, germinated at 25°C imbibition and 3°C imbibition	53
Table 2. Effect of stratification (moist chilling at 3°C) in germination and physiology of germinated seeds..	54
Figure 3. Accumulation of As and growth of germinants of 10-12 days.	55
Figure 4. Allometric relationship between total length and dry biomass, in germinants coming from exposition to five concentrations of As.	55
Figure 5. Ethylene production in germinants of 5 days and content of chlorophylls, carbohydrates, aminoacids and proteins in germinants of 10-12 days, under five concentrations of As during germination..	57
Figure 6. Responses of antioxidant enzymes and malate to five concentrations of As during the germination.	58
Table 3. Size, biomass, vigor and tolerance of seedlings (70 days) coming from control (25°C) and stratified (3°C) seeds germinated under five concentrations of arsenic.	58
Discussion.....	59
Conclusions.....	62
References.....	63
Appendices.....	69
C) CONCLUSÕES GERAIS	70

INTRODUÇÃO GERAL

Existem inúmeras associações estabelecidas ao nível global, entre altas concentrações de metais pesados e mineração. Elementos como cobre, cádmio, níquel, manganês, ferro, chumbo e zinco são liberados durante a extração de altos volumes de minerais do solo, especialmente com mineração a céu aberto. Porém, outros aspectos não tem sido igualmente pesquisados, como por exemplo a resposta do metaloide arsênio a este tipo de distúrbio ecológico, ou sim a mineração subterrânea gera os mesmos efeitos químicos no solo.

Na Colômbia, a mineração subterrânea de carvão é uma das principais atividades económicas, que é desenvolvida em zonas Andinas próximas aos cultivos de cebola, batata e ervilha. A necessidade de determinar se a mineração traz altas quantidades de metais pesados ou arsênio que possam ser mobilizadas às áreas cultivadas é urgente. O governo estabeleceu que as pessoas naturais ou jurídicas que realizem atividades de mineração devem, depois do abandono das minas, reflorestar para recuperar os solos afetados. Embora existe um desconhecimento sobre as espécies que possam ser usadas para estes planes de recuperação e os donos das minas preferem semear espécies exóticas como Pinus e Eucalyptus, que causam acidificação, redução da entomofauna benéfica e perda de retenção de água nos solos tropicais de montanha.

Esta pesquisa propõe atender três objetivos: (1) identificar quais elementos traça estão presentes nos solos pós-mina em concentrações potencialmente perigosas e quais espécies de árvores poderiam ter tolerância a essas condiciones químicas, (2) aprofundar no estudo das características germinativas de espécies potencialmente tolerantes, e gerar um protocolo que maximize a produção de plântulas e (3) estabelecer quais são os mecanismos fisiológicos ou bioquímicos que permitem a tolerância a metais pesados ou metaloides.

Esta pesquisa faz parte do projeto aprovado pela Fapemig (2016): Tolerância a metais pesados e efeitos ontogênicos em plantas: potencial para biorrecuperação de áreas afetadas por rejeitos de mineração, da Universidade Federal de Viçosa, e foi só o primeiro de um conjunto de pesquisas por parte do Laboratório de Biofísica Ambiental que atendem à problemática ecológica em torno da mineração para formular possíveis soluções.

CAPÍTULO 1
ARSENIC AND HEAVY METALS IN SOILS AFFECTED BY
COAL MINING IN SAMACÁ (COLOMBIA)

Arsenic and heavy metals in soils affected by coal mining in Samacá (Colombia)

Carolina Ramos^{1,3} M., Juraci A. de Oliveira², Mayer I. Lagos López³.

¹ Department of Plant Biology, Universidade Federal de Viçosa. Avenida PH Rolfs s/nº, Viçosa, Minas Gerais, Brazil.

² Department of General Biology, Laboratory of Biophysics, Universidade Federal de Viçosa. Avenida PH Rolfs s/nº, Viçosa, Minas Gerais, Brazil.

³ Escuela de Ciencias Biológicas, Universidad Pedagógica y Tecnológica de Colombia. Av. Central del Norte 39-115, Tunja, Colombia.

ABSTRACT

Coal mining is one of the most usual economic activities in the Andes region in Colombia. We sampled soils in the proximity of four coal underground mines in the municipality of Samacá and analyzed by ICP-AES the concentrations of the heavy metals aluminum (Al), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), zinc (Zn) and the metalloid arsenic (As). We employed a sequential extraction to separate the organic and exchangeable fractions of each element and single extractions to characterize the different phases of arsenic in soil. In addition, we traced 50m transects from the coal residuals toward the more undisturbed zone, in opposite direction to the mines, to describe plant composition and to evaluate change of diversity in intervals of 12.5m. The most frequent elements in the soils were Al ($1134.5 \pm 135 \text{ mg kg}^{-1}$), Fe ($1430.2 \pm 185.9 \text{ mg kg}^{-1}$) Mn ($45.96 \pm 7.9 \text{ mg kg}^{-1}$) and Zn ($47.22 \pm 7.21 \text{ mg kg}^{-1}$), but the exchangeable fraction of As, more available for uptake by plants, had potentially toxic concentrations ($7 \pm 0.35 \text{ mg kg}^{-1}$). The content of As in different species followed the order: Exchangeable > organic > bounded to oxides > leachable > bounded to carbonates. Overall, we found 68 species of plants distributed differently in relation with the site and distance to the coal source. In three sites, the indices of diversity Margaleff and alfa-Fisher near the mines were lower at larger

distances from the coal source. In the clusters of these three sites, the general composition of the distance 0-12.5m diverged from the rest of the intervals of distance. We found eight species that could have high tolerance to As because of being unique in the nearest zone of coal mines: *Alnus acuminata*, *Baccharis angustifolia*, *Baccharis latifolia*, *Camomila matricaria*, *Lolium multiflorum*, *Myrica parvifolia*, *Solanum lycioides* and *Trifolium repens*. The abundance of the generalist species *Penisetum clandestinum*, that was found at all intervals of distance from the source, was positive correlated with the concentration of As. Similarly, 13 species were only present at higher distances from the coal source (37.5-50m) what suggests that they are susceptible to pollution. We concluded that the underground coal mining in Samacá is related with potentially toxic concentrations of As, that must be immobilized to avoid its transport toward crop fields. For further studies, we propose a deeper research on the tolerance of *Alnus acuminata* to As, since they are the woody species that could be used for reforestation programs in post-mining soils.

Key words: Arsenic, diversity index, generalist, plant distribution, similarity.

INTRODUCTION

Coal is a fossil fuel widely used in the world. The best scenario of global recoverable resources projection estimates up to 1720 Gt of coal with a conversion energy of 31500 EJ, mainly concentrated in minerals of bituminous type (Mohr et al., 2015). Nevertheless, each step in the production chain of coal industry brings multiple hazards to the health, to the ecosystems and to the environment. During the extraction, toxic concentrations of the heavy metals Cr, Cu, Hg, Mn, Ni, Pb, Zn and the metalloid As become frequent in soils and water near coal mines. (Bhuiyan et al., 2010; Blindow et al., 1993; Fan et al., 2013; Lattuada et al., 2009; Qin et al., 2016).

Heavy metals and metalloids have innumerable effects on cell metabolism and plants physiology: replacement or interaction with essential elements and dysfunction of specific biomolecules, structural alteration of membranes, enzymatic inhibition and oxidative stress (Gratão et al., 2005; Yadav, 2010). Accordingly, those trace elements affect negatively the germination of seeds (Aydinalp and Marinova, 2009; Duarte et al., 2012; Sathy and Ghosh, 2013; Wani et al., 2012), photosynthesis and plant growth (Hattab et al., 2009; Mahdih et al., 2013; Päivöke and Simola, 2001; Yadav, 2010),

leading to changes in plant composition at the long time. Reduction in biodiversity associated to surface mining has been widely demonstrated (Antwi et al., 2008; Donggan et al., 2011; Li et al., 2011), but there are few evidences of similar effects in landscapes influenced by underground mining.

Arsenic is one of the pollutants with higher potential of toxicity considering that concentrations below 5 mg Kg⁻¹ are recommended for cultivated soils (Freeman, 1998; Golui et al., 2017; Tóth et al., 2016). The distribution and mobility of As in soils and plants depends on its chemical species. The inorganic form arsenate (V) is soluble in the water of the soil matrix, complexes easily with sulfur, aluminum and iron and is transported into the cells using the same way of phosphates. The reduced-inorganic ion arsenite (III) is the most mobile and toxic form of As despite being easily leachable. Some organic forms of As could be highly toxic, but these species have a low affinity for soils and sediments. (Adriano, 2001; Finnegan and Chen, 2012; Zhao et al., 2010).

At the ecosystem level, the relationship between the residuals of mining and the distribution of plants could be key to inferring the tolerance level of some species and to use that knowledge in restoration plans (Brunetti et al., 2009; Tamás and Kovács, 2005; Wong, 2003). Species with the capacity to immobilize, sequester or accumulate heavy metals are more frequent in the vicinity of contamination source and have a potential for soil recovery, while intolerant species will tend to disappear in the long time. The interactions between trace elements and physical and chemical properties of soils as acidity, organic matter and nitrogen content are also responsible, in some grade, of the plants distribution (Chandra et al., 2014; Wong, 2003).

The aim of this study was to assess the concentration of heavy metals and arsenic in soils affected by coal underground mining and to establish how the level of these pollutants could be related to plant diversity and composition. The expected goal was to find some native species that according to their distribution deserve a deeper research on their tolerance to soil pollutants and should be considered to use in recovering programs.

METHODS

Study Area

The research was conducted in the Municipality of Samacá, state of Boyacá, Colombia, where the natural ecosystems are the Andean paramo and the tropical high

mountain forests. A strong spatial-temporal heterogeneity in temperature and radiation favors the establishment of different types of vegetation: Tussock grasses, perennial herbs, giant rosettes, prostrate shrubs and small trees, with an important proportion of endemic taxa for the South American Andes (Rundel et al., 1998). The average of temperature in the region is 14.1°C and the annual precipitation is around 890 mm.

Four sites of abandoned coal mines were chosen for the sampling of soils and plants: Puente Burras – Salamanca (PBS) (5°27,950'N -73°31,778'W; 2857m), Cruz Colorada (CRC) (5°26,662'N -73°35,834'W; 3128m), Loma Redonda – Prodyser (LRP) (5°27,417'N -73°34,019'W; 3092m) and Milpa I Coralita – Mina la Limpia (MML) (5°28,107'N -73°33,327'W; 3184m). The locals of sampling were dispersed along a route of 11 Km in the mining zone. Sediments of arsenopyrite are distributed in the Andean region, and mining activities could release significant quantities of As in cultivated fields and superficial water. In Boyacá, maximum As levels of 40.7 mg kg⁻¹ were measured in soils, what could be related with a big mineral formation of bituminous and coking coal (Alonso et al., 2014). Underground coal mining for metallurgic industry in Boyacá is low tech and in many cases, illegal (Rudas and Hawkins, 2014). Therefore, there are not in the region efficient practices to avoid the runoff and leaching of pollutants toward cultivated areas of onion, potato and peas in the proximity of the mines. The government establish that mining companies must implement reforestation programs in post-mining soils but currently those programs involves majorly exotic tree species as pine and eucalyptus.

Sampling and analysis of Soils

From each site were taken four samples with approximately 500g of soil, in the contour of the coal residuals. Each sample was the result of the homogenization of four subsamples from 5 cm to 20 cm depth. The samples were dried at room temperature for 7 days and some physical and chemical properties were evaluated in the Laboratory of Soil Analysis: textural class, pH (water solution 1:1.25), effective cation exchange capacity (CEC), organic matter (OM), Al saturation, base saturation, P total by using Mehlich extractor and content of Mg⁺² and Ca⁺² by using KCl as extractor.

To determine the concentration of trace elements: Al, As, Cu, Fe, Mn, Ni, Pb and Zn, a sequential extraction of 1g of soil was performed (Jena et al., 2013; Tessier et al., 1979): (1) Shaking with 8mL of 1.0M MgCl₂ for 1 hour at room temperature (2) Addition of 3mL of 0.2M HNO₃ and 5mL of H₂O₂ for 3 hours with intermittent

agitation, at $85\pm 2^{\circ}\text{C}$, cooling and addition of 5mL of 3.2N ammonium acetate in 20% HNO_3 , continuous shaking for 30 minutes, centrifugation and removing the supernatant. At the end of each step, the solutions of soils were centrifuged and the respective supernatant was the extract for the exchangeable and organic (bioaccumulated or rizospheric) fractions. The extracts were analyzed by ICP-AES and the curves of calibration were constructed using multi-element standard.

Specific extractions for As

To detect another species of arsenic in the samples of soil, three additional single extractions were performed (Aguilar et al., 2007; Antosiewicz et al., 2008): (1) Addition of 1.0M Buffer phosphate pH 7.2 and shaking at 180 rpm for 14 hours, for the As linked to oxides of iron and aluminum; (2) Addition of EDTA pH 7.0 and shaking for 2 hours, for the As mobile and easily leachable; (3) Addition of 0.11M acetic acid pH 3.5 and shaking for 14 hours for the As linked to carbonates. Given that these fractions could be very lower than the total As detected by ICP, the extracts were analyzed by HG-AFS.

Vegetation sampling and analysis of biodiversity

One transect of 50 m and 4 m wide was established per site, starting from the source of coal and oriented toward the most undisturbed zone. All plants inside these transects were registered and directly identified. In some cases was necessary to take pictures to complete the identification. Transects were marked in four segments of 12.5m to carry out a comparative analysis among the closest and the farthest zones. The abundance (N) and richness (S) were used for the calculation of diversity indices: Shannon-Wiener entropy, Simpson's dominance and Pielou's evenness (J) (Jost, 2007; Keylock, 2005; Stirling and Wilsey, 2001). Since some herb species have a clonal growth, what causes an important distortion of the abundance, the total counting was standardized to a maximum value of 100.

Statistical analysis

The differences in the concentration of trace elements were tested through the software Statistica v.10 StatSoft, Inc. (2011), as well as the differences among the As

extracted in different phases. Pearson's correlations were used to establish associations between the concentration of trace elements and the abundance of wide-distributed species. The statistical analysis were performed with the software PAST v.1.98 (Hammer and Harper 2001) and Statistica v.10 StatSoft, Inc. (2011).

RESULTS

The content of Al, Cu, Fe, Mn Ni and Zn varied among sites and between phases, while the contents of As and Pb were less sensible to changes (Figure 1). The organic forms of Fe and Pb were highly dominant, contrary to As and Ni that were present mainly in exchangeable forms. In general, the soils of the study area presented heavy metals in the order: Fe ($1430.2 \pm 185.9 \text{ mg kg}^{-1}$) > Al ($1134.5 \pm 135 \text{ mg kg}^{-1}$) > Mn ($45.96 \pm 7.9 \text{ mg kg}^{-1}$) > Zn ($47.22 \pm 7.21 \text{ mg kg}^{-1}$) > As ($12.37 \pm 0.29 \text{ mg kg}^{-1}$) \geq Cu ($12.32 \pm 0.62 \text{ mg kg}^{-1}$) > Ni ($10.36 \pm 0.53 \text{ mg kg}^{-1}$) > Pb ($2.29 \pm 0.15 \text{ mg kg}^{-1}$). MML was the site with the most divergent concentrations of heavy metals in relation with the other sites, especially because it had a significant high organic content of Al, Cu, Fe, Mn and Zn in comparison with their exchangeable forms.

The additional extractions to detect other forms of As were efficient. In average the As leachable and bounded to oxides and carbonates contributed more than 10% to the total concentration (Figure 2). The proportion of As in different phases remained similar in all sites, except in the case of As bounded to Al/Fe oxides that reached almost 3 mg kg^{-1} in CRC. The content of the different species of As followed the order: Exchangeable ($7 \pm 0.35 \text{ mg kg}^{-1}$) > organic ($3.85 \pm 0.48 \text{ mg kg}^{-1}$) > bounded to oxides of Al / Fe ($1.32 \pm 0.23 \text{ mg kg}^{-1}$) > leachable ($0.2 \pm 0.09 \text{ mg kg}^{-1}$) > bounded to carbonates ($0.04 \pm 0.2 \text{ mg kg}^{-1}$).

The physical and chemical analysis showed that soils in the study area are clay loam, acids, low CEC, optimal content of OM and high Al saturation (Table1). There were found some associations between soil properties and concentration of trace elements. The OM was positively related with total Cu and the exchangeable Mn and Zn; the total As was negatively related with pH; the exchangeable Zn was negatively related with Al saturation and positively related with Ca^{+2} ; the exchangeable Cu was negatively related with the clay fraction. The organic fraction of heavy metals did not have any significant relationship with the physical and chemical properties of soil.

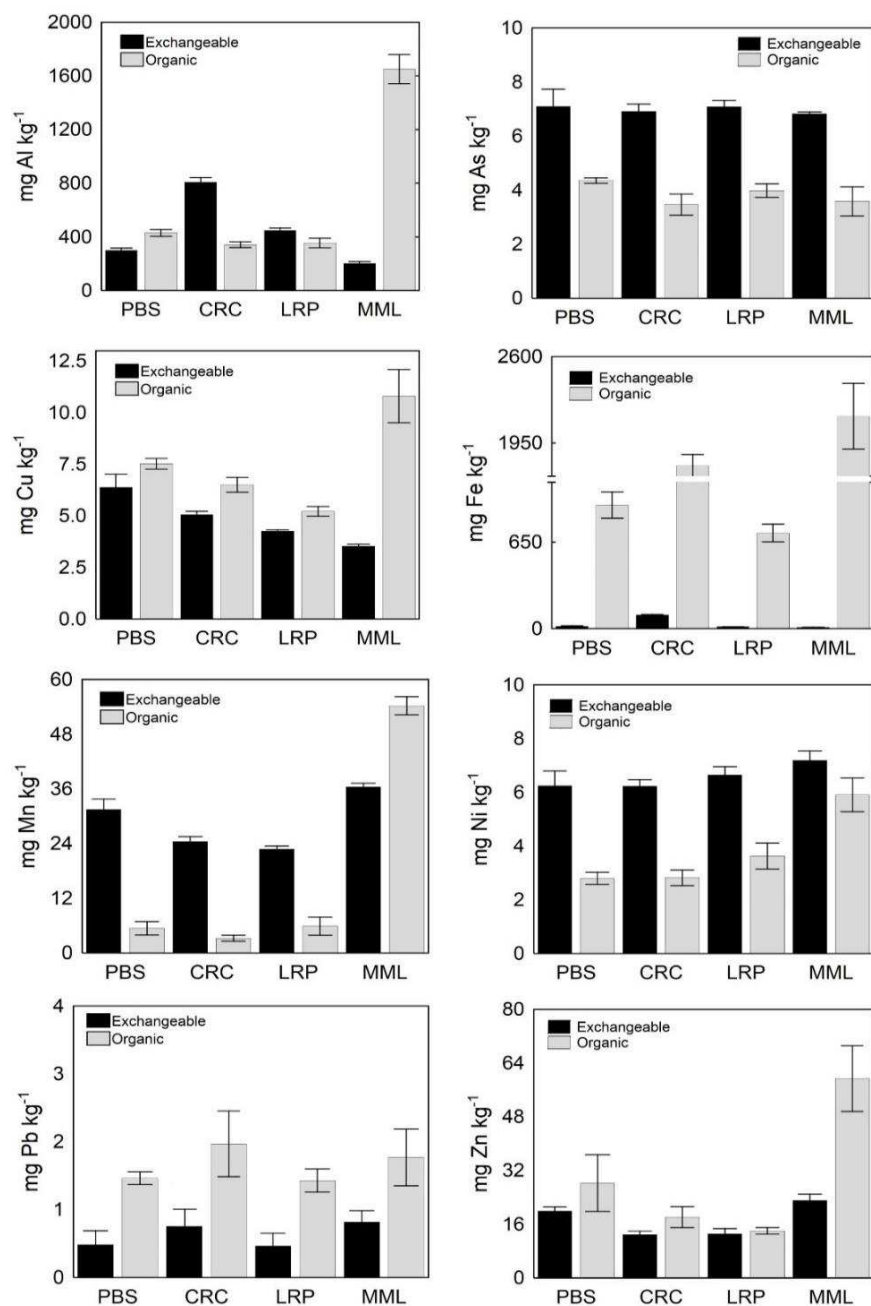


Figure 1. Exchangeable and organic content of heavy metals and the metalloid As in soils of four sites affected by coal mining. PBS: Puente Burras – Salamanca, CRC: Cruz Colorada, LRP: Loma Redonda, MML: Mina la Limpia. N=32, 4 replicates per extraction.

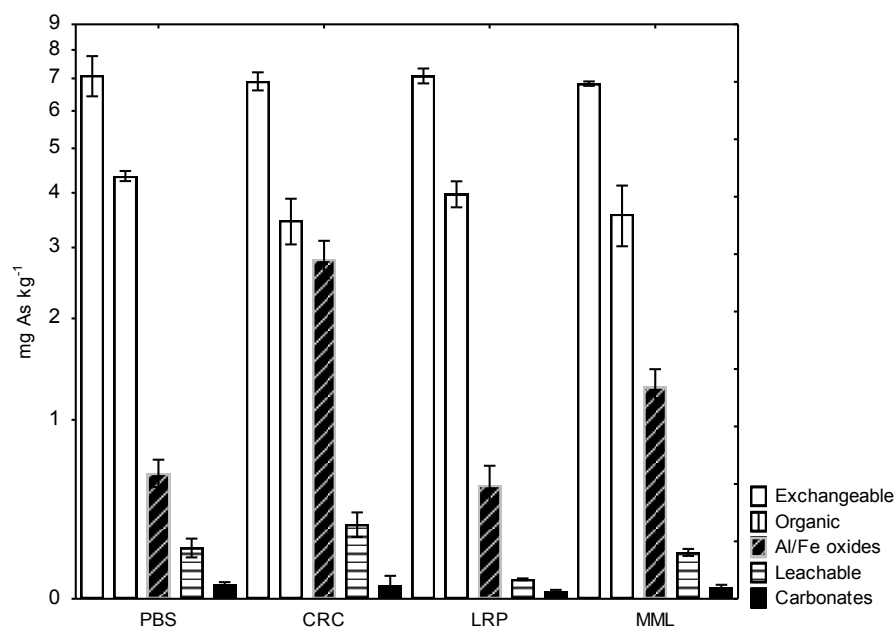


Figure 2. Speciation of As in soils of four sites affected by coal mining. N=80, 4 replicates per extraction.

Table 1. Physical and chemical properties of soils in the study area and associations with total (T) or exchangeable (EX) concentrations of trace elements.

	Max-Min values	Significant Pearson correlations (P<0.05)	
pH	3.12-3.88	-0.951	As _(T)
P	16.8-87.3 mg dm ⁻³	---	---
K	3-75 mg dm ⁻³	0.967	Ni _(EX)
CEC	9.01-13.16 cmol dm ⁻³	---	---
OM	2.68-8.96 dag kg ⁻¹	0.967	Cu _(T)
		0.993	Mn _(EX)
		0.974	Zn _(EX)
Ca ⁺²	0.82-5.41 cmol dm ⁻³	0.966	Zn _(EX)
Mg ⁺²	1.21-3.54 cmol dm ⁻³	---	---
Al saturation	20.5-81.9%	-0.983	Zn _(EX)
Base saturation	11.9-49.3 %	---	---
Clay fraction	34-54.6%	-0.959	Cu _(EX)
Sand fraction	14.1-31.6%	---	---
Silt fraction	18,7-34.5%	---	---

Overall, there were found 67 species of plants (Table 2): 12 species of trees, 22 species of shrubs, 31 species of herbs and grasses and two species of epiphytes. Their distribution showed some patterns in relation with the site and distance to the coal source: In three sites (PBS, CRM and LRP) the richness, diversity (H') and evenness (J) had a notable increase as the distance to the coal source also increased, and the contrary pattern was observed with the dominance (D) (Figure 3). The behavior of the diversity along the distance in the fourth site (MML) was different, despite having the highest increase of richness beyond of 20 m to the coal source.

The species *Hypochaeris radicata*, *Pennisetum clandestinum*, *Poa angustifolia* and *Rumex acetosella* have an extensive and clonal pattern of growth and were widely distributed in the study area, regardless the distance to coal mines. In general, CRC was the most diverse place ($S=34$, $N=330$, $H'=2.77$, 17 unique species) and PBS was the least diverse ($S=17$, $N=156$, $H'=2.023$, 8 unique species). Besides the already mentioned species, *Ageratina angustifolia*, *Camomila matricaria*, *Chusquea tessellata*, *Digitalis purpurea*, *Dodonea viscosa*, *Holcus lanatus*, *Lolium multiflorum*, *Munnozia senecionides* and *Orthrosanthum colombianum* were elements highly dominant in the herbaceous stratum. The representative neotropical trees were *Alnus acuminata*, *Miconia ligustrina* and *Macleania rupestris*. Eight species were present exclusively in the closest zone to the coal mine (0-12) but only one, *A. acuminata* was a woody species. Five species could be considered as generalist, since were present in every site and distance, and 13 species were only in the farthest zone to the coal mine (37.5-50m) (Table 2).

In three of the four sites: PBS, MML and CRC, the vegetation of the nearest zone to the coal source (0-12.5m) diverged from any other place more distant (12.5-50m) (Figure 4). The cluster analysis based in Euclidean distance showed that, excluding the segment 0-25m, the composition along the rest of the transect was consistently similar, highlighting the intrinsic diversity of sites. In LRP, the only place that did not show a differential composition in relation to the distance to the mine, the segment 0.0-12.5m was more similar to other segments of the same site.

Table 2. Plant species and corresponding abundance in four sites influenced by coal mining in Samacá (Colombia).

SPECIE	FAMILY	Distance to the coal source (m)			
		0-12.5	12.5-25	25-37.5	37.5-50
<i>Acacia melanoxyton</i>	Fabaceae	1	0	0	1
<i>Achyrocline bogotensis</i>	Asteraceae	5	0	2	0
<i>Ageratina angustifolia</i>	Asteraceae	0	0	3	10
<i>Ageratina colombiana</i>	Asteraceae	0	9	1	0
<i>Alnus acuminata</i>	Betulaceae	4	0	0	0
<i>Anagallis arvensis</i>	Primulaceae	0	1	0	0
<i>Baccharis angustifolia</i>	Asteraceae	2	0	0	0
<i>Baccharis latifolia</i>	Asteraceae	2	0	0	0
<i>Baccharis trinervis</i>	Asteraceae	1	2	0	0
<i>Bidens pilosa</i>	Asteraceae	0	0	0	2
<i>Buquetia glutinosa</i>	Melastomataceae	0	0	0	3
<i>Calamagrostis effusa</i>	Poaceae	12	0	3	1
<i>Camomila matricaria</i>	Asteraceae	16	0	0	0
<i>Carex sp.</i>	Poaceae	1	6	3	2
<i>Cestrum nocturnum</i>	Solanaceae	0	3	0	0
<i>Cestrum parvifolium</i>	Solanaceae	1	0	0	2
<i>Chusquea tessellata</i>	Poaceae	0	25	1	0
<i>Croton funckianus</i>	Euphorbiaceae	0	0	0	1
<i>Digitalis purpurea</i>	Plantaginaceae	1	15	10	0
<i>Diplostephium alveolatum</i>	Asteraceae	0	0	0	3
<i>Diplostephium jenesanum</i>	Asteraceae	1	1	2	0
<i>Dodonea viscosa</i>	Sapindaceae	8	5	2	2
<i>Erodium cicutarium</i>	Geraniaceae	0	0	0	3
<i>Eucalyptus globulus</i>	Myrtaceae	8	6	6	0
<i>Gaiadendron punctatum</i>	Loranthaceae	1	0	0	1
<i>Gynoxys miniphylla</i>	Asteraceae	0	4	9	4
<i>Hesperomeles goudotiana</i>	Rosaceae	0	2	5	1
<i>Holcus lanatus</i>	Poaceae	10	21	40	29
<i>Hypericum juniperinum</i>	Hypericaceae	0	13	15	14
<i>Hypochaeris radicata</i>	Asteraceae	41	14	31	14
<i>Juncus effusus</i>	Poaceae	0	1	0	0
<i>Lolium multiflorum</i>	Poaceae	28	0	0	0
<i>Macleania rupestris</i>	Ericaceae	0	0	4	0
<i>Miconia ligustrina</i>	Melastomataceae	3	2	2	0
<i>Monnina integrifolia</i>	Polygalaceae	0	1	0	0
<i>Monochaetum myrtoideum</i>	Melastomataceae	7	12	16	20
<i>Munnozia senecionides</i>	Asteraceae	2	5	0	16

SPECIE	FAMILY	Distance to the coal source (m)			
		0-12.5	12.5-25	25-37.5	37.5-50
<i>Myrcianthes leucoxylla</i>	Myrtaceae	0	2	1	0
<i>Myrica parvifolia</i>	Myricaceae	1	0	0	0
<i>Myrsine guianensis</i>	Myrsinaceae	0	0	0	1
<i>Orthrosanthum colombianum</i>	Iridaceae	7	13	1	1
<i>Oxalis triangularis</i>	Oxalidaceae	0	0	0	1
<i>Passiflora mixta</i>	Passifloraceae	0	0	1	2
<i>Pennisetum clandestinum</i>	Poaceae	26	27	23	24
<i>Pernettya prostrata</i>	Ericaceae	0	1	1	0
<i>Phytolacca bogotensis</i>	Phytolaccaceae	32	8	0	0
<i>Plantago rigida</i>	Plantaginaceae	0	0	0	5
<i>Poa angustifolia</i>	Poaceae	42	8	44	6
<i>Prunus serotina</i>	Rosaceae	0	0	0	1
<i>Pteridium aquilinum</i>	Dennstaedtiaceae	0	11	10	11
<i>Rubus ulmifolius</i>	Rosaceae	0	1	9	5
<i>Rumex acetosella</i>	Polygonaceae	11	19	27	43
<i>Salpichroa organifolia</i>	Solanaceae	0	0	1	0
<i>Solanum lycioides</i>	Solanaceae	1	0	0	0
<i>Solanum nigrum</i>	Solanaceae	0	0	0	1
<i>Sonchus lanatus</i>	Asteraceae	0	1	0	0
<i>Struthanthus quercicola</i>	Loranthaceae	0	0	1	0
<i>Taraxacum officinale</i>	Asteraceae	8	0	0	4
<i>Tillandsia biflora</i>	Bromeliaceae	0	0	0	1
<i>Tillandsia fendleri</i>	Bromeliaceae	0	0	0	1
<i>Trifolium pratense</i>	Fabaceae	37	0	0	5
<i>Trifolium repens</i>	Fabaceae	4	0	0	0
<i>Vaccinium meridionale</i>	Ericaceae	0	2	0	6
<i>Vallea stipularis</i>	Elaeocarpaceae	0	2	4	4
<i>Viburnum triphyllum</i>	Caprifoliaceae	0	4	10	5
<i>Weinmannia tomentosa</i>	Cunoniaceae	0	0	2	0
<i>Xylosma spiculiferum</i>	Salicaceae	0	0	0	1
TOTAL		324	247	290	257

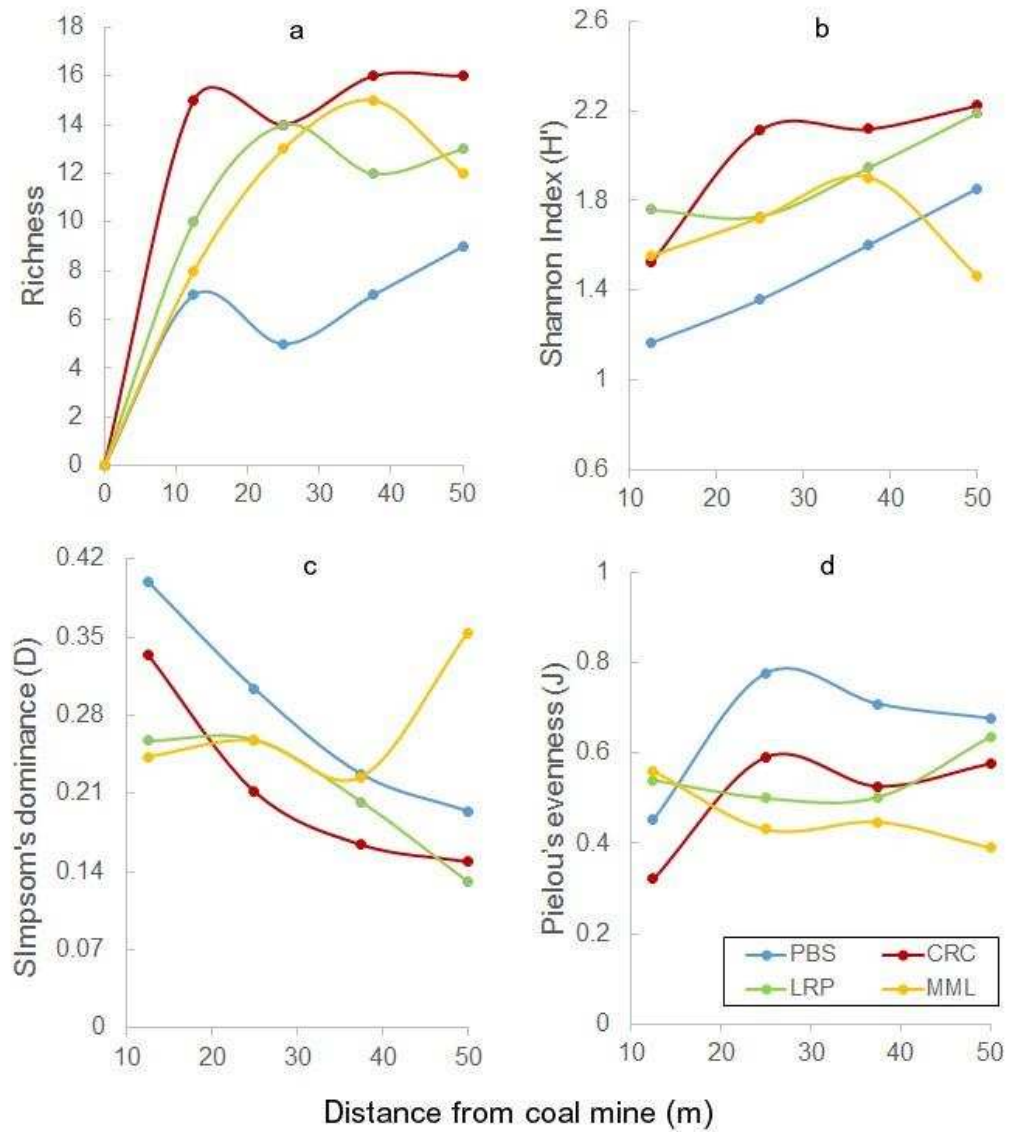


Figure 3. Changes in plant diversity from the coal residuals of mines toward 50 m of distance, in four sites of Samacá (Colombia). a.) Number of species; b) entropy; c) dominance; c) evenness. Values calculated for each 12.5 meters.

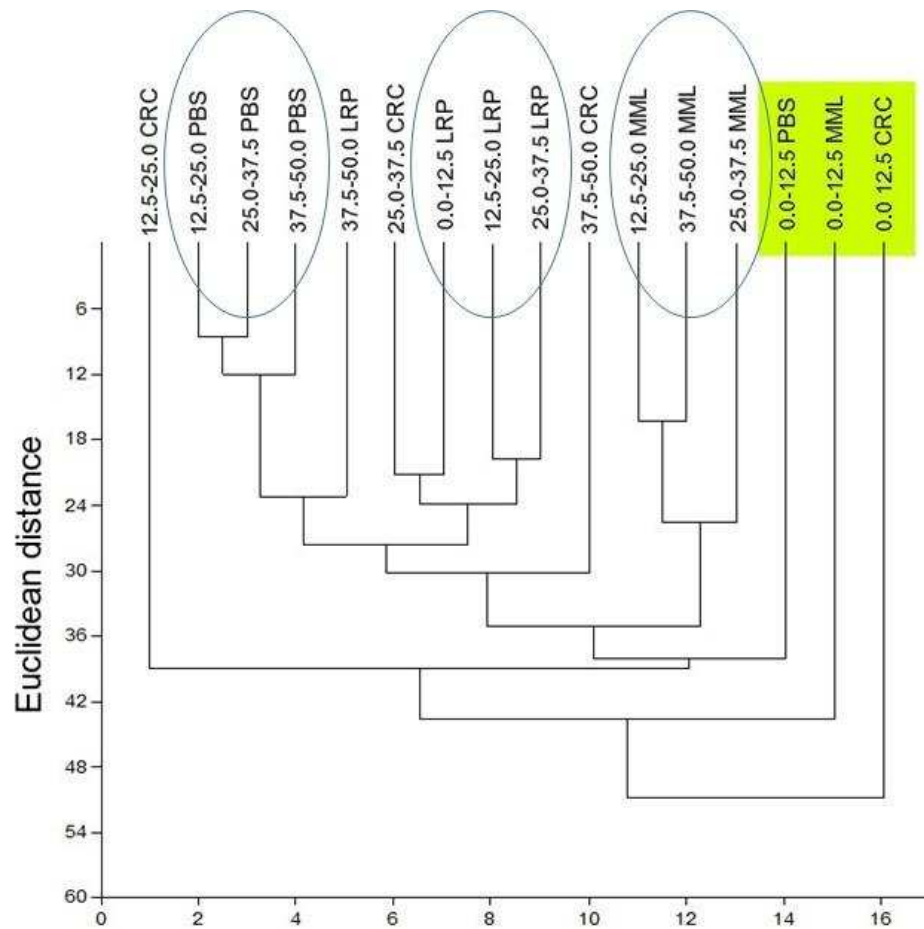


Figure 4. Cluster analysis of vegetation in the proximity of coal mine residuals. Notations of places have reference to the distance to coal source: 0-12.5 m, 12.5-25 m, 25-37.5 m and 37.5-50 m.

Five generalist species had sufficient abundance to explain variance associated to edaphic variables but only one, the herb *Pennisetum clandestinum*, had complete representation in the closest zone to the mines (0-12m), where were measured the soil variables. The abundance of *P. clandestinum* was contrasted with the 32 edaphic variables of heavy metals and metalloids (total content in their different phases) and physical or chemical properties of the soils. From all the 32 possible associations, the abundance of *P. clandestinum* only showed one significant correlation with the organic fraction of As ($y = 74.297x - 251.06$, $R^2 = 0.9745$, $N=4$).

DISCUSSION

In Colombia, reference values for heavy metal and metalloids in soils have not been established, but overall, the soils affected by coal mining in Samacá (Colombia) have contents of heavy metals that do not surpass the hazard levels established in different countries for agriculture (Nagajyoti et al., 2010; Rueda-Saa et al., 2011). Nevertheless, the concentrations of As measured in this study ($12.37 \pm 0.29 \text{ mg kg}^{-1}$) were higher than the average of As in the state of Boyacá (around 5 mg kg^{-1}) in floodplain of sediments of the Midwest and northeast of Boyacá (Alonso et al., 2014). There are several references about safe levels of As in soils below 10 mg kg^{-1} (Bundschuh et al., 2008; Freeman, 1998; Golui et al., 2017; Kabata-Pendias, 2010; Tóth et al., 2016). The results suggest that coal mining activity could be associated to the release of quantities of As potentially dangerous.

The analysis of the speciation of As (Figure 2) in the study area demonstrates that around 60% of the total As is exchangeable, the more available species for uptake by plants; however other studies have found that organic forms like MMA and DMA and inclusive the leachable As III are also absorbed by plants (Adriano, 2001; Li et al., 2015; Zhao et al., 2009). Furthermore, the negative correlation between As and pH suggest that releasing of arsenic in the superficial horizons could alter the chemical properties of soils. The acidic condition of these soils could favor the conversion of arsenate in arsenite, which is more toxic and mobile, then the contamination of cultivated areas in the proximity of the mines is a realistic scene. As an example, pea have a high rate of uptake As and negative effects in growth are evident at 8 mg As kg^{-1} (Päivöke and Simola, 2001).

The organic species were dominant in the case of the cationic elements Fe, Cu, Pb and Zn, as a result of the affinity to humic, fulvic and phytic acids in the rhizosphere (Giacalone et al., 2005; Jena et al., 2013). The positive correlations with organic matter are evidence of that affinity and probably a bio-accumulation at symbiotic level, e.g. micorhyzas, is involved (Mendoza et al., 2015). In the other side, the high saturation of Al had a negative relation with Zn, which is only one example of the capacity of Al to displace the cations from sites of adsorption at low pH (Table 1), reducing their availability (low base saturation) and affecting the plant nutrition.

Although each site had 32% to 50% of unique species, what gave them a biological identity, the vegetation in the study area was affected in its structure,

composition and diversity along transects that started in the coal source (mine and residuals) and ended at 50m of distance. In three of four sites the composition of the closest zones to the coal source was completely divergent in relation with the rest part of transects (Figure 4). In the same way, in three sites were demonstrated that richness, diversity and evenness increase as the distance from the coal mine increases, and the dominance of species is reduced as the distance to the mine increases (Figure 3).

These results showed that some edaphic variables associated with the coal mining affect the establishment and growth of the species, and since none of the heavy metals were in high concentration, the As is the only element potentially toxic in these soils. At concentrations of 10 mg L⁻¹ arsenic is able to reduce the germination and growth (Bleeker et al., 2006; Felipe, 2009; Duarte et al., 2012; González-Valdez et al., 2016) and its interactions with a high Al saturation and an extremely low pH, affects the plant diversity (Antwi et al., 2008; Chandra et al., 2014; Stoughton and Marcus, 2000).

Six species could be tolerant to trace elements because their presence (N>1) was limited to the closest zone to the coal mine (Table 2): *A. acuminata*, *B. angustifolia*, *B. latifolia*, *C. matricaria*, and *T. repens*. Among all of them, only *A. acuminata* is a woody species that should be studied more deeply to evaluate if it can be used in the reforestation planning demanded by the government. Among the generalist species, *P. clandestinum* showed a positive correlation with the organic fraction of As, and the nature of these relationship must be established. The first option is that *P. clandestinum* is provided with specific arsenic tolerance mechanisms. The second option, and more plausible, is the exclusion of As by release of organic exudates (Bergqvist et al., 2014). The third option is the mycorrhizal immobilization of As in the rhizosphere (Mendoza et al., 2015).

Finally, thirteen species were present at the higher distance from the coal source (37.5-50m) what suggests that they are highly susceptible to pollution and could not be considered in plans of postmining recovering: *B. pilosa*, *B. glutinosa*, *C. funckianus*, *D. alveolatum*, *E. cicutarium*, *M. guianensis*, *O. triangularis*, *P. rigida*, *P. serotina*, *S. nigrum*, *T. biflora* *T. fendleri* and *X. spiculiferum*.

CONCLUSIONS

Underground coal mining in Samacá does not release hazard quantities of heavy metals but generates potentially toxic concentrations of arsenic in the soil that must be immobilized to avoid its transport toward cultivated fields. Arsenic, alone or by interaction with the acidity of soils, is associated to significant reduction of plant diversity. It is recommendable a deeper research on the tolerance of *Alnus acuminata* to As, since it is the only woody species present at the closest zone to the coal residues, therefore it would be a key species for reforestation programs of post-mining soils.

REFERENCES

- Adriano, D.C., 2001. Trace elements in terrestrial environments: Biochemistry, Bioavailability and risks of metals, 2nd ed. Springer-Verlag, New York.
- Aguilar, J., Dorronsoro, C., Fernández, E., Fernández, J., García, I., Martín, F., Sierra, M., Simón, M., 2007. Arsenic Contamination in Soils Affected by a Pyrite-mine Spill (Aznalcóllar, SW Spain). *Water, Air, Soil Pollut.* 180, 271-281.
- Alonso, D.L., Latorre, S., Castillo, E., B.Brandão, P.F., 2014. Environmental occurrence of arsenic in Colombia: A review. *Environ. Pollut.* 186, 272-281.
- Antosiewicz, D.M., Escudé-Duran, C., Wierzbowska, E., Skłodowska, A., 2008. Indigenous Plant Species with the Potential for the Phytoremediation of Arsenic and Metals Contaminated Soil. *Water, Air, Soil Pollut.* 193, 197-210.
- Antwi, E.K., Krawczynski, R., Wiegleb, G., 2008. Detecting the effect of disturbance on habitat diversity and land cover change in a post-mining area using GIS. *Landscape Urban Plan.* 87, 22–32.
- Aydinalp, C., Marinova, S., 2009. The effects of heavy metals on seed germination and plant growth on alfalfa plant (*Medicago sativa*). *Bulg. J. Agric. Sci.* 15, 347-350.
- Bergqvist, C., Herbert, R., Persson, I., Greger, M., 2014. Plants influence on arsenic availability and speciation in the rhizosphere, roots and shoots of three different vegetables. *Environ. Pollut.* 184, 540-546.

- Bhuiyan, M.A.H., Parvez, L., Islam, M.A., Dampare, S.B., Suzuki, S., 2010. Heavy metal pollution of coal mine-affected agricultural soils in the northern part of Bangladesh. *J. Hazard. Mater.* 173, 384-392.
- Blindow, I., Andersson, G., Hargeby, A., Johansson, S., 1993. Long-term pattern of alternative stable states in two shallow eutrophic lakes. *Freshwater Biol.* 30, 159-167.
- Brunetti, G., Soler-Rovira, P., Farrag, K., Senesi, N., 2009. Tolerance and accumulation of heavy metals by wild plant species grown in contaminated soils in Apulia region, Southern Italy. *Plant Soil* 318, 285-298.
- Bundschuh, J., Giménez-Forcada, E., Guerequiz, R., Pérez-Carrera, A., Garcia, M.E., Mello, J., Deschamps, E., 2008. Fuentes geogénicas de arsénico y su liberación al medio ambiente, Distribucion del arsenico en las regiones Iberica e Iberoamericana. Editorial Programa Iberoamericano de Ciencia y Tecnologia para el Desarrollo, Buenos Aires, pp. 33-47.
- Chandra, R., Prusty, B.A.K., Azeez, P., 2014. Spatial variability and temporal changes in the trace metal content of soils: implications for mine restoration plan. *Environ. Monit. Assess.* 186, 3661-3671.
- Donggan, G., Zhongke, B., Tieliang, S., Hongbo, S., Wen, Q., 2011. Impacts of coal mining on the aboveground vegetation and soil quality: a case study of Qinxin coal mine in Shanxi Province, China. *Clean-Soil Air Water* 39, 219-225.
- Duarte, D.M., Gomes, M.P., Barreto, L.C., Matheus, M.T., Garcia, Q.S., 2012. Toxic trace elements effects on seed germination of four Brazilian Savanna tree species. *Seed Sci. Technol.* 40, 425-432.
- Fan, J., Sun, Y., Li, X., Zhao, C., Tian, D., Shao, L., 2013. Pollution of organic compounds and heavy metals in a coal gangue dump of the Gequan Coal Mine, China. *Chin. J. Geochem.* 32, 241-247.
- Finnegan, P.M., Chen, W., 2012. Arsenic toxicity: the effects on plant metabolism. *Front. Physiol.* 3, 182.
- Freeman, H., 1998. Standard handbook of hazardous waste treatment and disposal. McGraw-Hill, New York.
- Giacalone, A., Gianguzza, A., Orecchio, S., Piazzese, D., Dongarra, G., Sciarrino, S., Varrica, D., 2005. Metals distribution in the organic and inorganic fractions of soil: a case study on soils from Sicily. *Chem. Spec. Bioavailab.* 17, 83-93.

- Golui, D., Mazumder, D.N.G., Sanyal, S.K., Datta, S.P., Ray, P., Patra, P.K., Sarkar, S., Bhattacharya, K., 2017. Safe limit of arsenic in soil in relation to dietary exposure of arsenicosis patients from Malda district, West Bengal- A case study. *Ecotox. Environ. Safe.* 144, 227-235.
- Gratão, P.L., Polle, A., Lea, P.J., Azevedo, R.A., 2005. Making the life of heavy metal-stressed plants a little easier. *Funct. Plant Biol.* 32, 481-494.
- Hattab, S., Dridi, B., Chouba, L., Kheder, M.B., Bousetta, H., 2009. Photosynthesis and growth responses of pea *Pisum sativum* L. under heavy metals stress. *J. Environ. Sci.* 21, 1552-1556.
- Jena, V., Gupta, S., Dhundhel, R.S., Matic, N., Bilinski, S.F., Devic, N., 2013. Determination of total heavy metal by sequential extraction from soil. *Int. J. Res. Environ. Sci. Technol.* 3, 35-38.
- Jost, L., 2007. Partitioning Diversity into Independent Alpha and Beta Components. *Ecology* 88, 2427-2439.
- Kabata-Pendias, A., 2010. Trace elements in soils and plants. Taylor & Francis Group, Boca Raton, USA.
- Keylock, C.J., 2005. Simpson diversity and the Shannon–Wiener index as special cases of a generalized entropy. *Oikos* 109, 203-207.
- Lattuada, R.M., Menezes, C.T.B., Pavei, P.T., Peralba, M.C.R., Santos, J.H.Z.D., 2009. Determination of metals by total reflection X-ray fluorescence and evaluation of toxicity of a river impacted by coal mining in the south of Brazil. *J. Hazard. Mater.* 163, 531-537.
- Li, F., Liu, X., Zhao, D., Wang, B., Jin, L., Hu, D., 2011. Evaluating and modeling ecosystem service loss of coal mining: A case study of Mentougou district of Beijing, China. *Ecol. Complex.* 8, 139–143.
- Li, N., Wang, J., Song, W.-Y., 2015. Arsenic uptake and translocation in plants. *Plant Cell Physiol.* 57, 4-13.
- Mahdieh, S., Ghaderian, S.M., Karimi, N., 2013. Effect of arsenic on germination, photosynthesis and growth parameters of two winter wheat varieties in Iran. *J. Plant Nutr.* 36, 651-664.
- Mendoza, R.E., García, I.V., de Cabo, L., Weigandt, C.F., de Iorio, A.F., 2015. The interaction of heavy metals and nutrients present in soil and native plants with arbuscular mycorrhizae on the riverside in the Matanza-Riachuelo River Basin (Argentina). *Sci. Total Environ.* 505, 555-564.

- Mohr, S.H., Wang, J., Ellem, G., Ward, J., Giurco, D., 2015. Projection of world fossil fuels by country. *Fuel* 141, 120-135.
- Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M., 2010. Heavy metals, occurrence and toxicity for plants: a review. *Environ. Chem. Lett.* 8, 199-216.
- Päivöke, A.E.A., Simola, L.K., 2001. Arsenate Toxicity to *Pisum sativum*: Mineral Nutrients, Chlorophyll Content, and Phytase Activity. *Ecotox. Environ. Safe.* 49, 111-121.
- Qin, F.-x., Wei, C.-f., Zhong, S.-q., Huang, X.-f., Pang, W.-p., Jiang, X., 2016. Soil heavy metal(loid)s and risk assessment in vicinity of a coal mining area from southwest Guizhou, China. *J. Cent. South Univ.* 23, 2205-2213.
- Rudas, G., Hawkins, D., 2014. La minería de carbón a gran escala en Colombia : impactos económicos, sociales, laborales, ambientales y territoriales. Friedrich Ebert Stiftung, Bogotá.
- Rueda-Saa, G., Rodríguez, J.A., Madriñán-Molina, R., 2011. Metodologías para establecer valores de referencia de metales pesados en suelos agrícolas: Perspectivas para Colombia. *Acta Agron.* 60, 203-218.
- Rundel, P.W., Sharifi, M.R., Gibson, A.C., Esler, K.J., 1998. Structural and physiological adaptation to light environments in Neotropical *Heliconia* (Heliconiaceae). *J. Trop. Ecol.* 14, 789-801.
- Sethy, S.K., Ghosh, S., 2013. Effect of heavy metals on germination of seeds. *J. Nat. Sci. Biol. Med.* 4, 272-275.
- Stirling, G., Wilsey, B., 2001. Empirical Relationships between Species Richness, Evenness, and Proportional Diversity. *Am. Nat.* 158, 286-299.
- Stoughton, J.A., Marcus, W.A., 2000. Persistent impacts of trace metals from mining on floodplain grass communities along Soda Butte Creek, Yellowstone National Park. *Environ. Manage.* 25, 305-320.
- Tamás, J., Kovács, E., 2005. Vegetation pattern and heavy metal accumulation at a mine tailing at Gyöngyösoroszi, Hungary. *Zeitschrift für Naturforschung C* 60, 362-368.
- Tessier, P.C., Campbell, C., Bisson, M., 1979. Sequential Extraction Procedure for the Speciation of Particulate Trace Metals. *Anal. Chem.* 51, 844-851.
- Tóth, G., Hermann, T., Silva, M.R.D., Montanarella, L., 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. *Environ. Int.* 88, 299-309.

- Wani, P.A., Khan, M.S., Zaidi, A., 2012. Toxic Effects of heavy metals on germination and physiological processes of plants, in: Zaidi, A., Wani, P.A., Khan, M.S. (Eds.), Toxicity of Heavy Metals to Legumes and Bioremediation. Springer Vienna, pp. 45-66.
- Wong, M.H., 2003. Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. *Chemosphere* 50, 775-780.
- Yadav, S.K., 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *S. Afr. J. Bot.* 76, 167-179.
- Zhao, F., Ma, J., Meharg, A., McGrath, S., 2009. Arsenic uptake and metabolism in plants. *New Phytol.* 181, 777-794.
- Zhao, F.J., McGrath, S.P., Meharg, A.A., 2010. Arsenic as a Food Chain Contaminant: Mechanisms of Plant Uptake and Metabolism and Mitigation Strategies. *Annu. Rev. Plant Biol.* 61, 535-559.

APPENDICES

1. Coal mining zone from Samacá Colombia. Crops of onion and peas are in confluence with coal mines and coke ovens.



2. Plants growing on coal residuals.



3. Extraction of exchangeable and organic phases from soil samples collected in Samacá Colombia.



CAPÍTULO 2

EFECTO DE DIVERSOS FACTORES EN LA GERMINACIÓN Y DESARROLLO TEMPRANO DE *Alnus acuminata* Kunt (Betulaceae)

EFEECTO DE DIVERSOS FACTORES EN LA GERMINACIÓN Y DESARROLLO TEMPRANO DE *Alnus acuminata* Kunt (Betulaceae)

Carolina Ramos-Montaña^{1,2}, Juraci Alves de Oliveira² & Eduardo Fontes Araujo³

¹ Escuela de Ciencias Biológicas, Universidad Pedagógica y Tecnológica de Colombia, Av. Central del Norte 39-115, Tunja, Colombia. E-mail: carolina.ramos@uptc.edu.co.

² Laboratório de Biofísica, Departamento de Biologia, Universidade Federal de Viçosa, Avenida Peter Henry Rolfs, Campus Universitário, Viçosa-MG, Brasil. E-mail: jalves@ufv.br.

³ Laboratório de Sementes, Departamento de Fitotecnia, Universidade Federal de Viçosa, Avenida Peter Henry Rolfs, Campus Universitário, Viçosa-MG, Brasil. E-mail: efaraujo@ufv.br.

RESUMEN

Alnus acuminata es una especie arbórea recomendada para planes de recuperación de zonas degradadas en la región Andina. Sin embargo, sus capacidades germinativas han sido poco estudiadas y las investigaciones existentes muestran una gran variación en los porcentajes de germinación. En este estudio se evaluó el efecto del año de cosecha, la edad de la semilla, la estratificación, el sustrato, la temperatura y la luz en la germinación de *A. acuminata*, a través de diferentes experimentos combinados. La unidad experimental equivalente a una réplica en todos los casos fueron cajas de germinación de 10X10 cm con 0.1 g de semillas en papel germitest, excepto en el experimento que evaluaba el efecto del sustrato. Se compararon dos tipos de sustrato, germitest y arena-humus; dos tipos de temperatura: constante a 25°C y alterna 20-30°C; dos condiciones de luz: fotoperíodo 12/12h y oscuridad; y 5 tratamientos de estratificación húmeda a 4°C en oscuridad: 7, 15, 30, 45 y 60 días, y en un tratamiento combinado se evaluó la estratificación húmeda por 48 horas con y sin luz. Finalmente, para verificar el efecto de la luz se hizo seguimiento de 35 días a plántulas provenientes de semillas germinadas con y sin fotoperíodo. En el transcurso de los experimentos, fueron usadas semillas cosechadas en 2014, 2015 y 2016. Las semillas de *A. acuminata* presentaron una alta variabilidad germinativa entre cosechas y tiempo poscosecha, que

es acorde con los porcentajes de germinación reportados. La germinación máxima no superó el 30% y se cree que las semillas usadas en el experimento provenían de unos pocos árboles semilleros, con baja representatividad de semillas maduras durante la colecta. Las condiciones óptimas para germinación de *A. acuminata* fueron: almacenamiento a bajas temperaturas, pretratamiento de estratificación de pocos días, sustrato germitest y temperaturas alternas 20-30°C con fotoperiodo 12/12h. El efecto positivo de la luz fue verificado al comprobar que estimulaba la emergencia y expansión cotiledonar, el fototropismo positivo, el desarrollo de hojas verdaderas y la sobrevivencia de plántulas. Es así como se recomienda que en estudios posteriores el éxito germinativo sea evaluado mediante el número de plántulas viables.

Palabras clave: Cosecha, temperatura, estratificación, fotoperiodo, tiempo poscosecha.

INTRODUCCIÓN

El género *Alnus* es un grupo de árboles y arbustos comúnmente conocidos como alisos, representado en cuatro subgéneros de amplia distribución en Asia, Europa y el continente americano. Los registros palinológicos de *Alnus* son de más de 50 millones de años de antigüedad y aunque su expansión geográfica ha sido a lo largo del hemisferio norte, algunas especies se adaptaron a los ambientes tropicales descendiendo desde Norteamérica hasta Centro y Suramérica (Chen y Li, 2004). La madera del aliso es valorizada y pese a tratarse de árboles de bajo porte son un componente importante de los diversos ecosistemas de bosque, especialmente por su capacidad de fijar nitrógeno, lo que les da un considerable potencial de uso en sistemas agroforestales y planes de recuperación de suelos (Cordero y Boshier, 2003; Santi et al., 2013).

El aliso andino *Alnus acuminata* es un árbol que puede alcanzar hasta 30 m y es la especie de *Alnus* más representativa en Suramérica. Se distribuye en la cordillera de los Andes entre 1.500 hasta 3.000 metros de altitud, soportando periódicamente bajas temperaturas y estableciéndose en las zonas ribereñas, siendo un componente estructural del bosque de galería. *A. acuminata* es reconocida como una especie nativa de importancia para la reforestación de zonas degradadas en la región Andina, gracias a su sistema radicular extenso, regeneración en zonas abiertas, rápido crecimiento y capacidad de fijación de nutrientes (Murcia, 1997; Ospina et al., 2005; Castaño-Villa et al., 2014; Urgiles et al., 2014). Con relación a sus ventajas regenerativas, se ha

observado presencia de flores o frutos durante todo el año (Rojas Rodriguez y Gomez Córdoba, 2008). Cada kilogramo contiene entre dos y cuatro millones de semillas de *A. acuminata* que, a pesar de tener una pérdida rápida de viabilidad, dan lugar a 50.000 – 100.000 plántulas (Cordero y Boshier, 2003; Ospina et al., 2005).

Las semillas de *A. acuminata* miden entre 1.5 y 3 mm, y se forman en una estructura cónica dehiscente o estróbilo de brácteas duras, que cambia de color verde a marrón cuando está madura. Cada semilla consta del pericarpo, cobertura seminal y embrión, que ocupa la mayor parte de la semilla; las semillas de *Alnus* carecen de endospermo (Harrington et al., 2008). Características fisiológicas de las semillas, como la dormancia, la longevidad y la respuesta de la germinación a la luz varían entre especies del género *Alnus* y en el caso específico de *A. acuminata* aún han sido poco estudiadas.

Las flores masculinas y femeninas del aliso están presentes en el mismo individuo y la dispersión de semillas es anemófila. En general se ha reportado que en los estróbilos se produce una fracción considerable de semillas vacías, con porcentajes que varían dependiendo del lote (Harrington et al., 2008). En varias especies una óptima germinación requiere de pretratamientos de estratificación en frío, que pueden durar hasta 180 días, o del uso de temperaturas alternas en el germinador (Baskin y Baskin, 2001; De Atrip y O'Reilly, 2005; Gosling et al., 2009; Harrington et al., 2008; MAPA, 2009). Los escasos estudios de germinación en *A. acuminata* reportan resultados variables en los porcentajes de germinación, que van desde 1% hasta 80%, e indican una longevidad de las semillas menor a un año y un efecto positivo de la luz en la germinación (Ruiz y Orozco, 1986; Aceñolaza, 1997).

Dada la importancia de *A. acuminata* en los ecosistemas altoandinos, y el conocimiento limitado con relación a sus patrones germinativos, que es un obstáculo para el desarrollo de metodologías que mejoren el potencial de uso de la especie en planes forestales y de restauración, el presente estudio se enfocó en el efecto de diferentes factores sobre la germinación de *A. acuminata*. Con el fin de descubrir cómo el año de cosecha, la edad de la semilla, la estratificación, el sustrato, la temperatura y la luz participan en la gran variabilidad de germinación reportada, y establecer cuáles son las condiciones óptimas que maximizan la germinación.

METODOLOGÍA

Procedimiento base

Todas las semillas de *A. acuminata* usadas durante los experimentos fueron suministradas por la empresa forestal El semillero S.A. Durante la realización del estudio fueron usadas semillas cosechadas en diciembre de 2014, junio de 2015 y febrero de 2016. La información del embalaje indicó una pureza promedio del 60%, y el pretratamiento recomendado por la empresa era de mínimo 24 horas de imbibición en agua a temperatura ambiente antes de colocar las semillas a germinar. Las semillas fueron almacenadas a 4°C y 35% de humedad relativa hasta el experimento correspondiente.

En total se realizaron cinco experimentos de germinación. El procedimiento base para la mayoría de esos experimentos consistió en colocar las semillas totalmente sumergidas en agua desionizada durante 48 horas en frascos de 30 ml protegidos de la luz. Luego 0.1 ± 0.005 g de las semillas preimbibidas fueron colocadas en cajas de germinación estándar de 10X10 cm con el sustrato correspondiente. A excepción del experimento 2, en todos los casos el sustrato germinativo fue papel germitest en doble capa, humedecido con unos 6 ml de agua (2.5 veces el peso del papel). Las cajas fueron llevadas a cámara de germinación para ajustar las condiciones específicas de luz, temperatura y fotoperiodo para cada experimento. La luz dentro de la cámara provenía de cuatro lámparas fluorescentes de 35 vatios, que al estar encendidas generaban una radiación aproximada de $50 \mu\text{mol m}^{-1} \text{s}^{-1}$. Cada caja de germinación con semillas fue definida como una réplica, y se consideró una semilla germinada cuando la radícula emerge a través del pericarpo. El número de semillas germinadas fue registrado cada tres o cuatro días hasta estabilización de la curva y con esa misma periodicidad fue aleatorizada la posición de las cajas dentro de la cámara de germinación. Se hizo reposición de agua en las cajas a lo largo del seguimiento.

Experimento 1: Efecto de la cosecha en la germinación

Seis réplicas de cada lote de semillas: 2014, 2015 y 2016 fueron colocados a germinar a temperatura constante de 25° y sin luz.

Experimento 2: Efecto de la cosecha, temperatura y sustrato en la germinación

Semillas del lote 2014 y semillas del lote 2015 fueron sometidas a germinación bajo dos condiciones de temperatura: Constante a 25°C y alterna 20-30°C (12/12 h). Adicionalmente fueron evaluados dos tipos de sustrato: mezcla orgánica de arena-humus en proporción 1:1 y papel germitest. El diseño final fue de tres factores con 4 réplicas.

Experimento 3: Efecto de la edad de la semilla en la germinación.

Semillas de la colecta de 2015 con diferentes tiempos poscosecha: 35, 50, 80 y 95 días, fueron colocadas a germinar bajo temperaturas alternas de 20-30 en oscuridad. Se evaluó la germinación en 4 réplicas.

Experimento 4: Efecto de la estratificación húmeda en la germinación

Semillas de la colecta de 2015 fueron sometidas a diferentes niveles de estratificación. Las semillas control recibieron el pretratamiento base de imbibición a 25°C por 48 horas. Las semillas sometidas a estratificación fueron sumergidas en agua desionizada y mantenidas en refrigeración a 4°C durante 7, 15, 30, 45 y 60 días, tiempo al final del cual fueron colocadas en cajas de germinación. Se registró la germinación en 6 réplicas por tratamiento, bajo condiciones de temperatura alterna 20-30°C (12/12h) sin luz.

Experimento 5: Efecto de la luz y la estratificación.

Para este experimento se activó el fotoperíodo 12/12 h en la cámara de germinación con temperaturas alternas 20-30°C y para las semillas sin fotoperíodo se cubrieron las cajas de germinación en plástico negro. Semillas del lote 2015 fueron sometidas a cuatro tratamientos: Control (preimbibición a 25° por 48 horas) con y sin fotoperíodo, estratificación (preimbibición a 4°C por 48 horas) con y sin fotoperíodo. Se montaron 5 réplicas para cada uno de los cuatro tratamientos.

Experimento 6: Efecto de la luz en el desarrollo temprano

Este experimento tenía la finalidad de evaluar si el número de germinados era el mejor estimador del éxito germinativo de los tratamientos, especialmente en el caso de la luz, que tiene un efecto regulatorio más prolongado durante la germinación y el desarrollo temprano, o si el número de plántulas normales es el mejor evaluador de la

germinación. Los primeros 24 germinados de los 2 tratamientos: Estratificación con fotoperíodo y estratificación sin luz, provenientes del experimento 5, fueron separados y mantenidos sobre papel germitest humedecido a temperatura de 25°C y fotoperíodo 12/12 durante 10 días, tiempo en el cual se evaluó:

La emergencia cotiledonar: Porcentaje de germinados donde los cotiledones expulsaron la envoltura seminal, pero aún permanecen cerrados.

La expansión cotiledonar: Porcentaje de germinados con cotiledones abiertos y orientados en posición perpendicular con el eje de crecimiento.

El fototropismo positivo: Porcentaje de germinados que se orientan en posición vertical en dirección a la luz, con cotiledones en máxima exposición. En este estado de desarrollo los germinados ya presentan una diferenciación clara entre la zona caulinar y la zona radicular.

Una vez evidente la diferenciación radicular, los germinados fueron trasladados para bandejas con arena estéril permanentemente humedecida, donde el seguimiento se extendió por dos semanas más para evaluar otros tres eventos de desarrollo: La aparición de la primera hoja verdadera con una medida mínima de 2 mm, la presencia de dos o más hojas verdaderas y la sobrevivencia en el día 35 desde la germinación. La presencia de hojas verdaderas fue considerada como la característica que demarca el paso de germinado a plántula.

Análisis estadístico

Los datos de germinación fueron corregidos por la pureza y expresados en porcentaje. Inicialmente se verificó la distribución normal de resultados de los experimentos 1 a 5 con el test de Kolmogorov-Smirnov y la probabilidad de Lilliefors. Luego se comparó los efectos de los diferentes factores, independientes o en posible interacción, mediante análisis de varianza y comparación múltiple de medias de Fisher LSD. En el caso del experimento 6 los resultados fueron expresados en frecuencia relativa (%) pero el análisis estadístico aplicado fue un test de Chi cuadrado de homogeneidad (X^2) para determinar si las distribuciones de las frecuencias en los múltiples eventos de desarrollo son iguales con o sin luz. Todas las pruebas estadísticas fueron desarrolladas con el software Statistica v.10.0 (StatSoft, Inc.).

RESULTADOS

Efecto de la cosecha, temperatura y sustrato en la germinación

Bajo condiciones de temperatura constante, la media de germinación del 2014 ($19.74 \pm 2.25\%$) fue mayor a la del 2015 ($13.13 \pm 2.77\%$), que a su vez fue mayor a la del 2016 ($5.33 \pm 0.53\%$) (Figura 1). Cuando las semillas de los lotes 2014 y 2015 fueron germinadas en oscuridad, las temperaturas alternas $20-30^{\circ}\text{C}$ estimularon significativamente la germinación en comparación con la temperatura constante a 25°C , alcanzando $28.54 \pm 3.04\%$ en el lote 2014 y $19.8 \pm 0.74\%$ en el lote 2015. Además las semillas en temperaturas alternas germinaron en un tiempo medio de 24 días, mientras que las semillas a 25°C demoraron 30 días en germitest y 42 días en sustrato orgánico. El papel germitest fue el mejor sustrato para la germinación de *A. acuminata*, pues en sustrato orgánico a 25°C la germinación se redujo en 37% y 49% para 2014 y 2015 respectivamente (Figura 2).

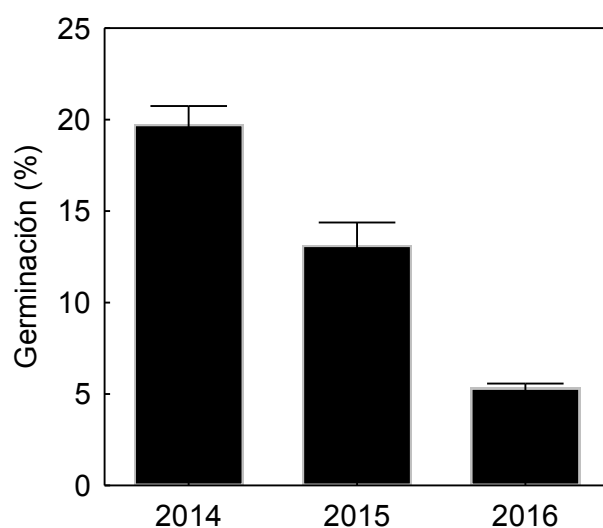


Figura 1. Germinación de *A. acuminata* en tres cosechas diferentes, bajo condiciones estándar de 25°C y fotoperíodo 12/12 h.

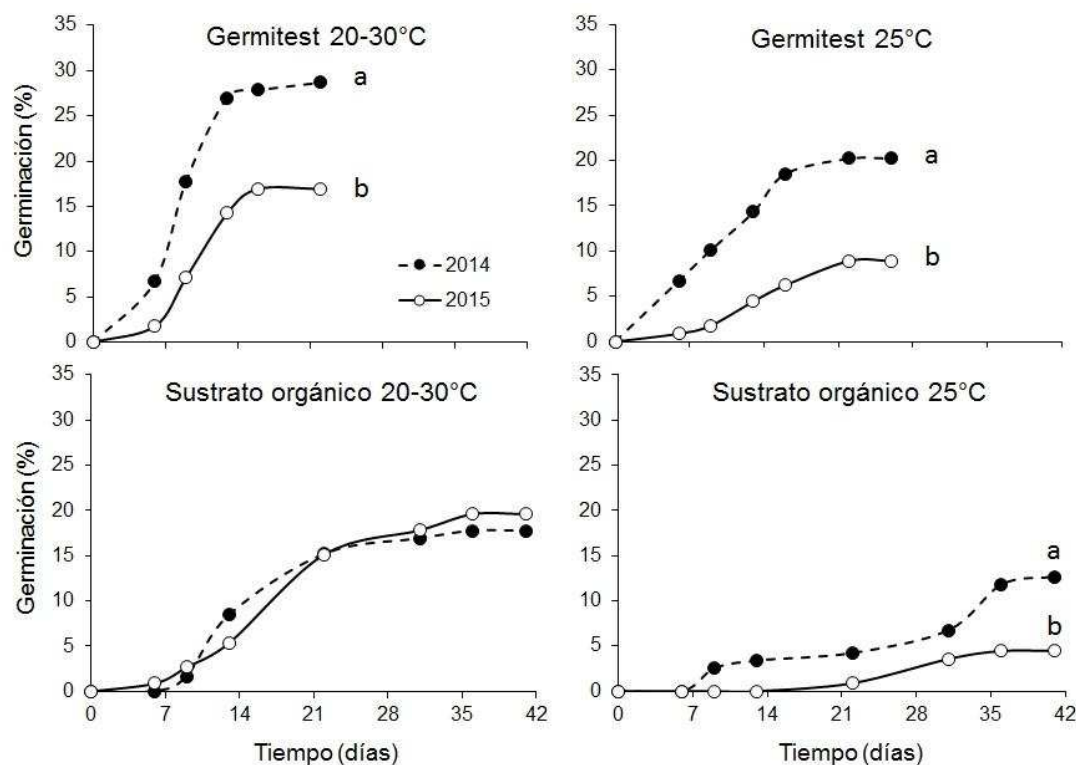


Figura 2. Efecto de la temperatura y el sustrato en semillas de *A. acuminata* de cosecha 2014 y 2015, en condiciones de oscuridad.

Efecto del tiempo poscosecha en la germinación.

La germinación de *A. acuminata* varió con el tiempo de almacenamiento. Inicialmente fue notorio que las semillas de 35 días no presentaron un patrón germinativo típico sigmoideal, con dos períodos de aceleración de la germinación y retraso en el tiempo de estabilización. Las semillas con mayor tiempo poscosecha si mostraron un mejor ajuste al patrón típico sigmoideal, con una fase de estabilización alcanzada hacia el día 25. La germinación promedio de semillas de 80 días (25.02%) fue significativamente mayor a las germinación en los demás tiempos poscosecha ($18.66 \pm 1.08\%$) (Figura 3).

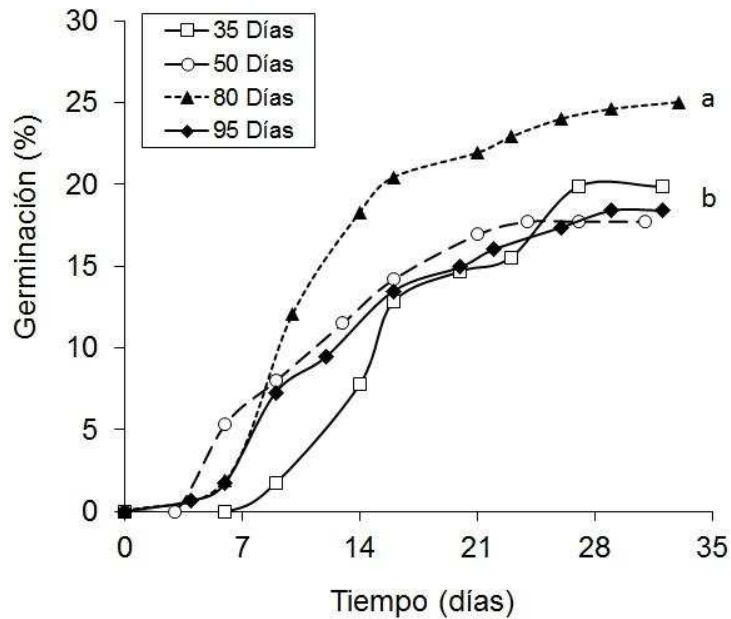


Figura 3. Efecto de la edad de diferentes semillas (tiempo de almacenamiento) en la germinación de *A. acuminata*. Condiciones de germinación: Temperatura alterna 20-30°C, sin luz.

Efecto de la estratificación húmeda en la germinación

La estratificación a 4°C no tuvo efectos positivos en la germinación de semillas en condiciones de oscuridad y temperaturas alternas 20-30°C (Figura 4). Los pretratamientos de 7 y 15 días de estratificación dieron una germinación similar al control (germinación promedio: $21.7 \pm 3.9\%$) pero al aumentar los tiempos de pretratamiento la germinación fue cada vez menor: 11.9%, 5.2% y 1.83% con 30, 45 y 60 días, respectivamente.

Efecto de la luz y la estratificación

Las semillas con mayor germinación fueron aquellas que recibieron estratificación húmeda de 48 horas con fotoperiodo ($29.25 \pm 1.7\%$) y el tratamiento menos eficiente fue la imbibición normal a 25°C en condiciones de oscuridad ($19.8 \pm 2.1\%$) (Figura 5). Al comparar los tratamientos con el mismo tipo de imbibición, fue notorio que el efecto de la luz llevó a una mayor germinación en ambos casos, aunque el efecto de la luz no fue estadísticamente significativo (Efecto de la estratificación: $F=12.25$, $p<0.01$. Efecto de la luz: $F=0.64$, $p=0.43$).

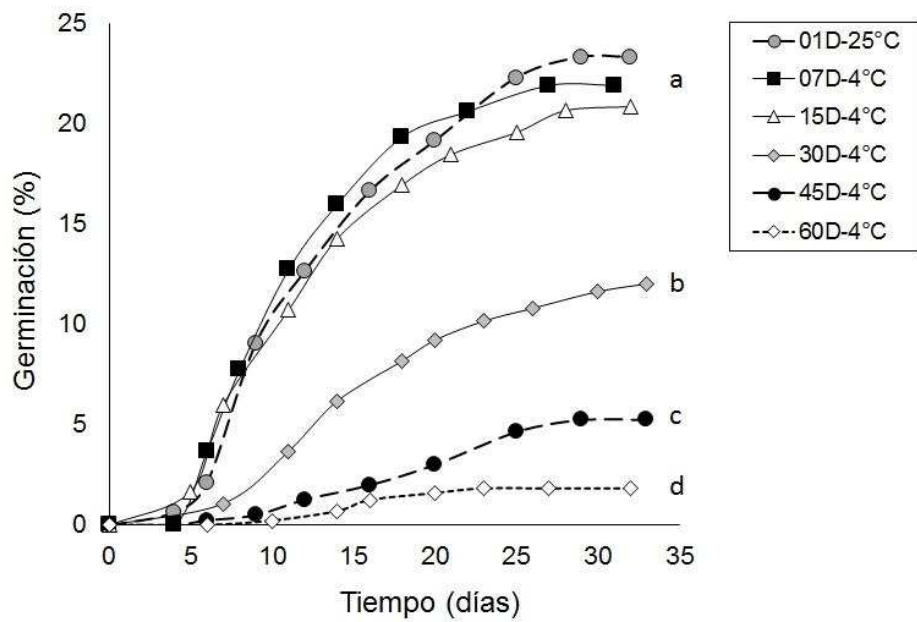


Figura 4. Efecto de estratificación húmeda en frío (4°C) de 7, 15, 30, 45 y 60 días, en la germinación de *A. acuminata*. Condiciones de germinación: Temperaturas alternas 20-30°C, sin luz.

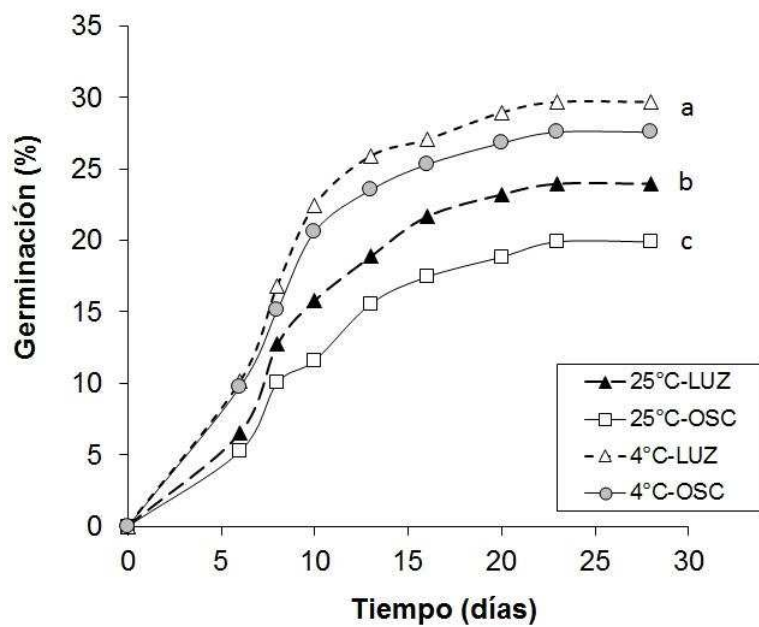


Figura 5. Efecto del pretratamiento de estratificación húmeda a 4°C versus preimbibición a 25°C, en la germinación de semillas con y sin fotoperíodo de 12/12 horas. Temperatura: Alterna 20-30°C.

Efecto de la luz en el desarrollo temprano

La luz tuvo una influencia positiva en todos los parámetros de desarrollo temprano. Las plántulas que provenían de semillas germinadas en oscuridad presentaron una frecuencia menor de emergencia cotiledonar (-12.5%), expansión cotiledonar (-12.5%), fototropismo positivo (-16.6%), aparición de la primera hoja verdadera (-25.5%), presencia de dos o más hojas verdaderas (-20.1%) y sobrevivencia (-34.5%), a lo largo de un seguimiento de 35 días (Figura 6). La diferencia en las distribuciones de la respuesta múltiple fue altamente significativa ($X^2=21.89$, g.l=5, $p<0.01$).

DISCUSIÓN

En este estudio *A. acuminata* presentó una germinación menor al 30% y diferencias significativas entre años de cosecha, rasgo que no es común en las especies del género *Alnus*. Se han reportado porcentajes de germinación inferiores al 50% en *Alnus glutinosa*, *Alnus incana*, *Alnus serrulata* y *Alnus viridis* (Harrington et al., 2008). Sin embargo, para el caso específico de *A. acuminata* la germinación encontrada es suficiente para asegurar la producción de más de 100.000 plántulas por kilo de semillas.

Los resultados también demostraron la alta variabilidad germinativa reportada para *A. acuminata*, que se ha sugerido estar relacionadas a diferencias en el potencial germinativo entre árboles semilleros, e incluso en un mismo árbol semillero. En un estudio en Guatemala se comparó la germinación de semillas provenientes de 50 árboles de una misma población y se encontró valores entre 2.5% y 94% (Murillo, 1998) mientras en un estudio similar en Argentina se evaluó 10 árboles semilleros con valores de germinación entre 24% y 79% (Aceñolaza, 1997). Se ha demostrado que esa amplia variación en la capacidad de germinación de *A. acuminata* está relacionada además con el tamaño del estróbilo y la posición que la semilla ocupa en el estróbilo (Ruiz y Orozco, 1986).

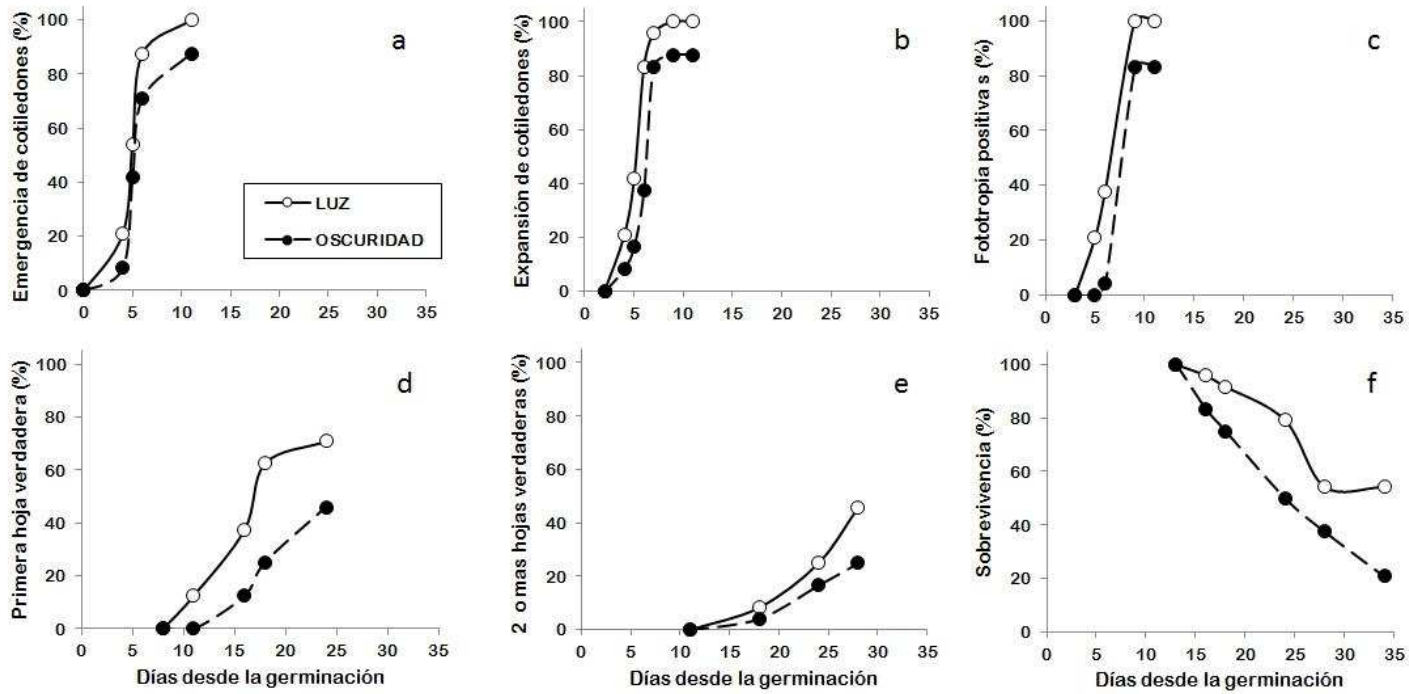


Figura 6. Efecto de la luz durante la germinación, en el desarrollo hasta plántula fotosintética con 2 o más hojas verdaderas, a lo largo de 35 días de seguimiento en fotoperiodo 12/12 h. Los porcentajes corresponden a la frecuencia relativa de un N inicial de 24 germinados.

La variación germinativa interanual y por edad de la semilla se ajusta a especies anemófilas que producen muchas semillas, dando lugar a diferentes fracciones: semillas vacías, semillas inmaduras, semillas viables que germinan y semillas dañadas. Estos rasgos exponen algunas similitudes en el comportamiento germinativo de *Alnus* y de especies coníferas (Kroiss and HilleRisLambers, 2015; Daskalidou et al., 2017).

La germinación de semillas de *A. acuminata* de 80 días poscosecha fue significativamente mayor a la de semillas de 35 o 50 días poscosecha, lo que sugiere que la semilla tiene dormancia. Previamente se había reportado que la capacidad germinativa de aumenta con un tiempo de almacenamiento de 20 días y que aparentemente una parte de las semillas alcanza la madurez durante ese período (Ruiz y Orozco, 1986). En casos similares con otras especies se ha considerado que el almacenamiento a temperaturas frías es per se una estratificación seca (Cousins et al., 2014). Sin almacenamiento a bajas temperaturas la viabilidad de las semillas de *Alnus* se reduce significativamente en unos pocos meses (Aceñolaza, 1997; Baskin and Baskin, 2001). En este estudio se observó que las semillas más antiguas, colectadas en el 2014 y almacenadas a bajas temperaturas aún tenían conservaban su capacidad germinativa a mediados del 2016.

Se comprobó con los diferentes experimentos que las mejores condiciones para germinar las semillas de *A. acuminata* son: papel germitest como sustrato, temperaturas alternas 20-30°C y condiciones de luz 12/12 h. La germinación también fue favorecida por un pretratamiento de imbibición a 4°C por 48 horas, que indicaría que las semillas de *A. acuminata* tienen algún grado de dormancia.

Al igual que *A. acuminata*, existen varias especies de *Alnus* que tienen su germinación óptima con temperaturas alternas 20-30°C (Baskin y Baskin, 2001; MAPA, 2009) lo que se atribuye a los contrastes climáticos que estas plantas experimentan en su área de distribución y que determinan la época favorable para la germinación. *A. acuminata* soporta una alta variabilidad diurna de temperatura, típica de los ecosistemas altoandinos, que se amplía especialmente en la época seca, justamente la época recomendada para la colecta de semillas (Ospina et al., 2005). No obstante, la máxima germinación registrada en este estudio se dio en el experimento 4 donde las temperaturas alternas 20-30°C eran combinadas con luz y estratificación húmeda a 4°C.

En un estudio comparativo de especies arbóreas en un mismo ecosistema, se encontró una especie con interacción positiva de luz y temperatura alterna, otra con

efecto positivo de la temperatura alterna independiente de la luz, otra con efecto positivo de la luz y poco dependiente de la temperatura, y una última especie de rápida germinación y totalmente irresponsiva a luz o temperatura (Vargas Figueroa et al., 2015). O que muestra que las especies presentan diferenciación de sus estrategias regenerativas para asegurar su coexistencia. Otros estudios han sugerido que las temperaturas alternas compensan la germinación de semillas fotoblásticas en ausencia de luz, mientras que la luz no tiene el mismo efecto compensatorio en semillas que requieran de oscilaciones de temperatura (Simão y Takaki, 2008). Aunque en este estudio no se evaluó el efecto de la luz de manera independiente, ya existen reportes de que a 25°C la luz cuadruplica la germinación de semillas de *A. acuminata* en comparación con condiciones de oscuridad (Araya et al., 2000).

La preimbibición a 4°C o estratificación húmeda aumentó la germinación de semillas de *A. acuminata* en condiciones de fotoperiodo 12/12 h, y de acuerdo con la clasificación de tipos de dormancia (Baskin y Baskin, 2004) estos rasgos corresponden a semillas con una dormancia morfofisiológica, que sería superada con aplicación de giberelina. Los efectos positivos de la aplicación de esta hormona en semillas de *A. acuminata* y *A. glutinosa* ya han sido documentados (Araya et al., 2000; De Atrip y O'Reilly, 2007) con la diferencia de que *A. glutinosa* presenta óptimos de germinación con 30 días de estratificación. Las especies *A. crispa* y *A. virilis* también tienen tratamientos recomendados de estratificación hasta de 60 y 90 días respectivamente (Harrington et al., 2008).

Finalmente, la producción de plántulas normales es en este caso el mejor estimador del éxito germinativo. Mientras el experimento 5 no encontró efectos directos de la luz en la germinación, el experimento 6 demostró que la presencia de luz durante la germinación es fundamental en todos los pasos del desarrollo temprano hasta llegar al estado de plántula autónoma con mínimo dos hojas verdaderas. Al final de 35 días de seguimiento, la supervivencia hasta este estado de plántula era del 54% en comparación con sólo el 21% cuando las semillas habían germinado en oscuridad. Dado que esta especie inicia simbiosis con *Frankia* y micorrizas a temprana edad (Cordero y Boshier, 2003; Becerra et al., 2009), con el sustrato adecuado estas plántulas de 35 días ya tienen opciones favorables de crecimiento. Ensayos similares que evalúen el éxito germinativo mediante el número de plántulas viables son recomendables para verificar los efectos de otros factores ambientales en *A. acuminata*.

CONCLUSIONES

Las semillas de *A. acuminata* presentan una alta variabilidad germinativa entre cosechas y edad de la semilla, que explicarían a su vez la alta variabilidad en los porcentajes de germinación reportados. Las condiciones óptimas para germinación de *A. acuminata* fueron: almacenamiento a bajas temperaturas, pretratamiento de estratificación de pocos días, sustrato germitest y temperaturas alternas 20-30°C con fotoperiodo 12/12 h. Además se recomienda evaluar el éxito germinativo con el conteo de plántulas viables.

REFERENCIAS

- Aceñolaza, P.G.** (1997) Longevidad y variación en los porcentajes de germinación de *Alnus acuminata* Kunth ssp *acuminata* (Betulaceae). *Brenesia*, 109-115.
- Araya, E., Gómez, L., Hidalgo, N. and Valverde, R.** (2000) Efecto de la luz y del ácido giberélico sobre la germinación in vitro de Jaúl (*Alnus acuminata*). *Agronomía Costarricense*. 24, 75-80.
- Baskin, C.C. and Baskin, J.M.** (2001) *Seeds: ecology, biogeography, and evolution of dormancy and germination*. San Diego, USA, Academic Press.
- Baskin, J.M. and Baskin, C.C.** (2004) A classification system for seed dormancy. *Seed Science Research*. 14, 1-16.
- Becerra, A.G., Menoyo, E., Lett, I. and Li, C.Y.** (2009) *Alnus acuminata* in dual symbiosis with *Frankia* and two different ectomycorrhizal fungi (*Alpova austroalnicola* and *Alpova diplophloeus*) growing in soilless growth medium. *Symbiosis*. 47, 85-92.
- Castaño-Villa, G.J., Estevez, J.V. and Fontúrbel, F.E.** (2014) The role of native forest plantations in the conservation of Neotropical birds: The case of the Andean alder. *Journal for Nature Conservation* 22, 547-551.
- Cordero, J. and Boshier, D.H.** (2003) *Árboles de Centroamérica: un manual para extensionistas*. Costa Rica, CATIE.

- Cousins, S., Witkowski, E. and Mycock, D.** (2014) Seed storage and germination in *Kumara plicatilis*, a tree aloe endemic to mountain fynbos in the Boland, south-western Cape, South Africa. *South African Journal of Botany*. 94, 190-194.
- Chen, Z. and Li, J.** (2004) Phylogenetics and biogeography of *Alnus* (Betulaceae) inferred from sequences of nuclear ribosomal DNA ITS region. *International Journal of Plant Sciences*. 165, 325-335.
- Daskalaku, E.N., Koutsovoulou, K., Mavroei, L., Tsiamitas, C., Kafali, E., Radaiou, P.-E., Ganatsas, P. and Thanos, C.A.** (2017) Interannual variability of germination and cone/seed morphometric characteristics in the endemic Grecian fir (*Abies cephalonica*) over an 8-year-long study. *Seed Science Research*, 1-10.
- De Atrip, N. and O'Reilly, C.** (2005) Effect of seed moisture content during prechilling on the germination response of alder and birch seeds. *Seed Science and Technology*. 33, 363-373.
- De Atrip, N. and O'Reilly, C.** (2007) Germination response of alder and birch seeds to applied gibberellic acid and priming treatments in combination with chilling. *Annals of Forest Science*. 64, 385-394.
- Gosling, P.G., McCartan, S.A. and Peace, A.J.** (2009) Seed dormancy and germination characteristics of common alder (*Alnus glutinosa* L.) indicate some potential to adapt to climate change in Britain. *Forestry*. 82, 573-582.
- Harrington, C.A., Brodie, L.C., DeBell, D.S. and Schopmeyer, C.S.** (2008) *Alnus* P. Mill.: Alder. pp 232-242 in Bonner, F.T. and Karrfalt, R.P. (Eds) *Woody plant seed manual*. Washington, USA, Department of Agriculture, Forest Service.
- Kroiss, S.J. and HilleRisLambers, J.** (2015) Recruitment limitation of long-lived conifers: implications for climate change responses. *Ecology*. 96, 1286-1297.
- MAPA** (2009) *Regras para análise de sementes*. Brasilia, Brasil, Ministério da Agricultura, Pecuária e Abastecimento de Brasil.
- Murcia, C.** (1997) Evaluation of Andean alder as a catalyst for the recovery of tropical cloud forests in Colombia. *Forest Ecology and Management*. 99, 163-170.
- Murillo, O.** (1998) Variación en parámetros de germinación de una población natural de *Alnus acuminata* de Guatemala. *Boletín Mejoramiento Genético y Semillas Forestales (CATIE)*, 4-8.
- Ospina, C.M., Hernández, R.J., Gómez, D.E., Godoy, J.A., Aristizábal, F.A., Patiño, L.N. and Medina, J.A.** (2005) *Guías silviculturales para el manejo de*

especies forestales con miras a la producción de madera en la zona andina colombiana: El Aliso o Cerezo. Manizales Colombia, CENICAFÉ.

Rojas Rodriguez, F. and Gomez Córdoba, G. (2008) Árboles del Valle Central de Costa Rica: reproducción. Revista Forestal Mesoamericana Kurú. 5, 2008.

Ruiz, M.C. and Orozco, M. (1986) Efecto de la posición de los frutos en el estróbilo y tamaño de los mismos, sobre el porcentaje de germinación en *Alnus acuminata* H.B.K. (Betulaceae). Caldasia. 14, 455-463.

Santi, C., Bogusz, D. and Franche, C. (2013) Biological nitrogen fixation in non-legume plants. Annals of botany. 111, 743-767.

Simão, E. and Takaki, M. (2008) Effect of light and temperature on seed germination in *Tibouchina mutabilis* (Vell.) Cogn.(Melastomataceae). Biota Neotropica. 8, 63-68.

Urgiles, N., Strauß, A., Loján, P. and Schübler, A. (2014) Cultured arbuscular mycorrhizal fungi and native soil inocula improve seedling development of two pioneer trees in the Andean region. New forests. 45, 859-874.

Vargas Figueroa, J.A., Duque Palacio, O.L. and Torres González, A.M. (2015) Germinación de semillas de cuatro especies arbóreas del bosque seco tropical del Valle del Cauca, Colombia. Revista de Biología Tropical. 63, 249-261.

APÉNDICES

1. Cajas de germinación listas para colocar semillas de *A. acuminata*.



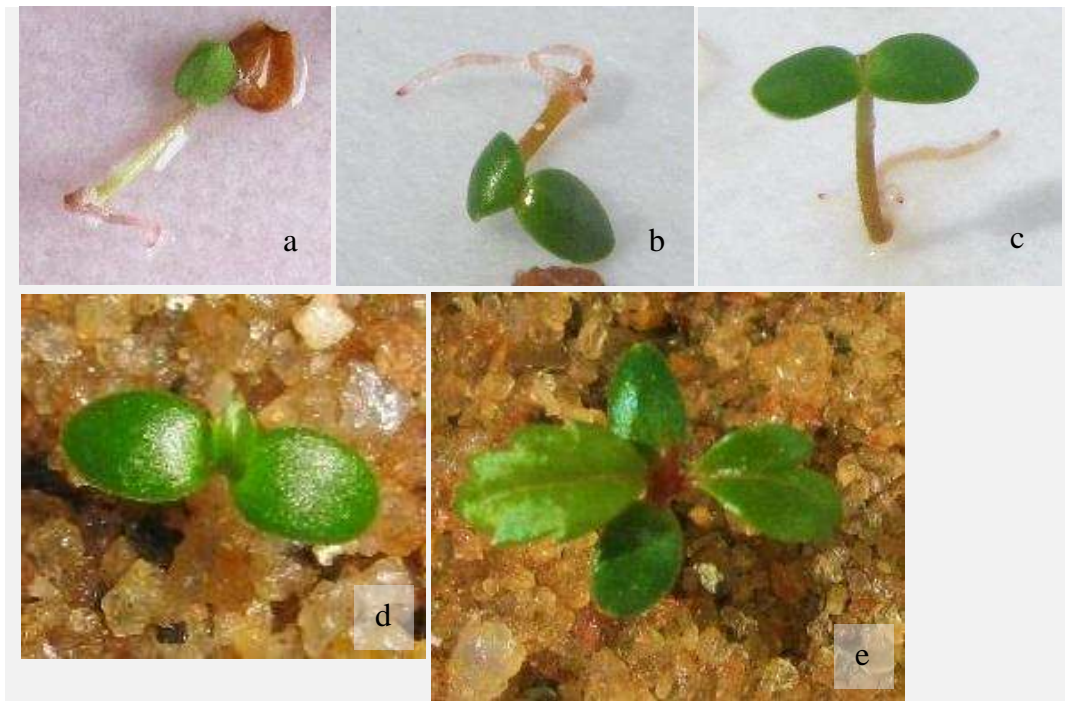
2. Semillas imbibidas de *A. acuminata*. Cada marcación en la regla a la derecha corresponde a 1 mm.



3. Semillas de *A. acuminata* germinadas en oscuridad (izquierda) y con fotoperiodo 12/12 h (derecha), correspondientes al experimento 6. Nótese una mayor expansión cotiledonar y fototropismo positivo en el segundo caso.



4. Seguimiento de germinados a lo largo de 30 días a partir de la germinación. a. Emergencia cotiledonar. b. Expansión cotiledonar. c. Fototropismo positivo. d. Aparición del primordio foliar. e. Plántula con dos hojas verdaderas.



CAPÍTULO 3

**MOIST PRE-CHILLING IMPROVES THE GERMINATION TOLERANCE OF THE
ANDEAN TREE *Alnus acuminata* TO ARSENIC**

MOIST PRE-CHILLING IMPROVES THE GERMINATION TOLERANCE OF THE ANDEAN TREE *Alnus acuminata* TO ARSENIC

Carolina Ramos-Montaña^{1,3}, Juraci Alves de Oliveira², Jorge A. Condori³ Afpata

¹ Escuela de Ciencias Biológicas, Universidad Pedagógica y Tecnológica de Colombia., Tunja, Colombia.

² Department of General Biology, Laboratory of Biophysics, Universidade Federal de Viçosa. Minas Gerais, Brazil.

³ Unidade de Crescimento de Plantas, Department of Plant Biology, Universidade Federal de Viçosa. Viçosa, Minas Gerais, Brazil.

Abstract

This research evaluated the effect of chilling imbibition in the germination and vigor of the Andean tree *Alnus acuminata*, at five concentrations of arsenic (As). Boxes of germination with 0.1 g of seeds preimbibed for 48 hours at 25°C (control) and 3°C were incubated at alternating temperatures 20-30°C and photoperiod of 12 hours. The concentration of As applied in the boxes were 0.0, 2.5, 5.0, 7.5, and 10 mg L⁻¹. The germination, imbibition, and ethylene were evaluated, and the biochemical, morphological and physiological traits of germinants of 10-12 days were analyzed to understand the changes caused by pretreatment and As during the early development. Five germinants of each combined treatment were grown until the day 70, and the final measures of size and biomass were taken to calculate the vigor index and the tolerance index. The chilling imbibition enhanced the germination tolerance of *A. acuminata* to As and this effect was unaltered by As concentration. The most susceptible point in the early development was the apparition of the first leaf that was delayed in germinants coming from seeds imbibed at 25°C and exposed to As. Germination of seeds imbibed at 3°C was 50% higher than in seeds without prechilling. Despite accumulating high amounts of As (34-68 µg g⁻¹ dry weight), the maximum reduction of growth was around 28% at 2.5 and 7.5 mg L⁻¹, but these germinants continue growing until the day 70, when the monitoring

finalized without differences in size or biomass with control plants. The biochemical and antioxidant traits of germinants suggest that accumulation of As in cotyledons, a high content of carbohydrates, the activity of SOD and POX and probably the synthesis of phytochelatins are the best strategies that could explain the tolerance of *A. acuminata* to As. In general, chilling preimbibition enhances the vigor and tolerance in more than 55%, and this treatment becomes indispensable for the implementation of *A. acuminata* in forestry programs in soils affected by coal mining.

Key Words: Antioxidant enzymes; early development; seeds; stratification; tolerance index, vigor index.

Introduction

Arsenic (As) is a metalloid naturally present in the lithosphere, in minerals of arsenopyrite, and therefore potentially present in plants at very low concentrations. However, human activities as mining and use of agrochemicals could release significant quantities of As in the soil, allowing toxic levels and its transference to the food chain (Adriano, 2001; Bundschuh et al., 2008).

Adverse effects of As in non-tolerant plants have been found since the initial stages of development. During the seed imbibition, arsenate (As^V) incomes to the cell through channels of phosphate, and given the chemical similarity of both ions the arsenic replace the structural position of phosphorous in different molecules, especially in the nucleic acids (Zhao et al., 2009; Finnegan and Chen, 2012). The entry of the arsenite (As^{III}) is through NIP-type aquaporines and causes reduction of the content of proteins and inhibition of the metabolism of carbohydrates (Isayenkov and Maathuis, 2008; Sanal et al., 2014). In general, 50 mg $As.L^{-1}$ are enough to reduce the germination in more than 50% (Talukdar, 2011; Duarte et al., 2012; Mahdih et al., 2013; Srivastava et al., 2013). At lower concentrations, seedlings present reduction in number of leaves, growth of root and stem, biomass and content of pigments (Felipe et al., 2009; Moreno-Jiménez et al., 2009; Imran et al., 2015).

The tolerance to As is common in plants growing in polluted environments. Different cellular and biochemical mechanisms are the external immobilization by exudates or symbiotic associations, the blocking of the apoplastic influx, the chelation and sequestration of As in specific cell structures, the activity of antioxidant enzymes and the elimination of arsenite (Song et al., 2010; Bothe, 2011; Del-Toro-Sánchez et al., 2013; Chao et al., 2014). Nevertheless, there is a scarce knowledge about the tolerance to As and the associated biochemical changes in seeds or germinants.

In the other side, the cold stratification, that is the pre-treatment of seeds at cold or chilling temperatures, is one of the most usual methods to improve the germination of seeds with some grade of dormancy. Moist prechilling have been tested with optimum results in many tree species, but in most of cases the effect of prechilling depends on its duration and the temperatures of germination (Bourgoin and Simpson, 2004; Rawat et al., 2008; Shahi-Gharahlar et al., 2012; Houšková and Martiník, 2015). In the model plant *Arabidopsis thaliana* cold stratification is related with changes in the type and synthesis of aquaporins at the later stage of germination, increases the transcription of α expansins, and induces the expression of alcohol dehydrogenases (Weitbrecht et al., 2011). All these evidences demonstrate that stratification not only acts in the breaking of dormancy, but also promotes the germination.

Apparently, besides the positive effect on germination, the cold stratification could improve the tolerance to stressing conditions. Stratified seeds of the grass *Leymus chinensis* have a better germination under drought and saline conditions than non-treated seeds (Lin et al., 2017), and a previous study had demonstrated that embryos of *Brassica napus* cultivated in vitro enhance their tolerance to desiccation when stratified at 4°C for 10 days (Anandarajah et al., 1991). The evaluation of the relation between stratification and the tolerance to As is a new issue that deserves to be addressed.

Alnus acuminata is an Andean tree with important potential for reforestation in degraded soils. The presence of this species in the proximity of coal mining zones in Boyacá-Colombia suggest some level of tolerance to As and its germination is optimized by a moist prechilling followed by incubation at alternating temperatures of 20-30°C with 12 hours of photoperiod. In this study, the germination tolerance of *A. acuminata* to As was evaluated, with and without a moist chilling pretreatment. The main objectives was

to describe the biochemical and physiological changes that occur under arsenic influence, during germination and early development, and to establish if cold stratification improves germination, vigor and tolerance.

Materials and Methods

Chilling moist pretreatment, Leaking and Germination

Seeds of *A. acuminata* used in this study were collected in June of 2016, provided by a forestall company. They were stored at 4°C and 50-52% of relative humidity until the germination tests that started in August of 2016. Control seeds were preimbibed in dark vials of 30 mL by 48 hours in deionized water at room temperature of 25°C and chilled seeds were preimbibed in the same conditions but at 3°C. Each experimental unity was a plastic germination box of 10x10 cm with 0.1 ± 0.005 g of preimbibed seeds on germitest paper.

Five solutions of As were prepared with deionized water and arsenate (Merck; $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$): 0.0 mg As L⁻¹, 2.5 mg As L⁻¹, 5.0 mg As L⁻¹, 7.5 mg As L⁻¹ and 10 mg As L⁻¹. The application of 14 mL of solution was randomly assigned to germination boxes, given in total 10 combined treatments of preimbibition and As with four replicates per treatment. These boxes were sprinkled with Captan 3 g L⁻¹ (Sigma-Aldrich) for fungi controlling and incubated in a chamber with 12 hours of photoperiod, alternating temperatures of 20-30°C. Germination were registered each four days until day 28. All the procedure was repeated as many times as necessary to produce enough germinants for the next assays.

Early development of germinants

The germinants of 12-15 days were washed with distillated water and separated in glasslock containers with moist germitest paper, where the early development was monitored: cotyledon emergency, cotyledon expansion, appear of the first leaf (what was considered the transition point between germinant and seedling), and presence of two or more leaves. All these events were registered as percentage from the initial number of

germinants. Also, the diameter of cotyledons, the length of roots and stems, and the fresh and dry weight were measured.

Determination of arsenic in germinants

Germinants of 12-15 days were dried at 85°C for 48 hours and 10 mg of material were digested with 5 ml of a nitric-perchloric solution (5:2 HNO₃ 65%, HClO₄ 70%) (Soon, 1998). Samples were left overnight in the solution and heated slowly for almost 1 hour at 95±5°C until total release of nitrogen; then the temperature were elevated to 130±5°C for more than two hours until release of white fume. The samples were filtered and the final volume was completed to 10 mL. Determination of As was made in five replicates per treatment by HG-AFS (fluorescence spectrometer PSA10.055) and the As atomic absorption standard solution of Sigma was used for curve calibration.

Biochemical analysis

The ethylene emission of seeds pre-imbibed for 48 hours (1 g of seeds per vial, five replicates) and germinants (25 germinants per vial, four replicates) was measured after 20 hours of incubation of the sealed vials at 25°C in constant light. The emission of ethylene was quantified by gas chromatography.

There was collected 25±2 mg of fresh weight of germinants (11-14 individuals) per eppendorf. The material was transported in liquid nitrogen and conserved at -85°C until biochemical analysis. In the laboratory the tissues were grinded and solved in 250 µL of ethanol 95% (v/v), heated at 80°C for 20 minutes and centrifuged at 12000 g for 5 minutes. The supernatant were transferred into a new eppendorf and the pellet was resuspended for two additional cycles of extraction, with ethanol 80% and 50%, respectively. The mixture of supernatant (around 680 µL) was used immediately for determination of chlorophylls a and b (Porra et al., 1989) and stored at -20°C. The pellet was homogenized with 400 µL of KOH 0.1M, heated at 95°C for 1 hour and stored at -20°C.

The supernatant was used for determination of aminoacids by color reaction with ninhydrin (Cross et al., 2006) and determination of glucose, fructose and sucrose by reading the NADPH produced by enzymatic activity of hexokinase, phosphoglucose isomerase and invertase, respectively (Fernie et al., 2001). For determination of malate

the activity of malate dehydrogenase was measured (Nunes-Nesi et al., 2007). The supernatant of the eppendorf with pellet was used for determination of protein with Bradford reagent (Cross et al., 2006). Subsequently, the pellet was hydrolyzed with acetic acid and starch was quantified by measuring the NADPH produced by activity of hexokinase and glucose-6-phosphate dehydrogenase (Ferne et al., 2001).

Antioxidant activity

A solution for enzymatic extraction was prepared with the following components and final concentrations: buffer of potassium phosphate 100 mM pH 7.0, EDTA 0.1 mM, PMSF 1 mM and DTT 1 mM. Approximately, 50 mg of fresh weight of germinants (23-28 individuals) were triturated with 600 μ L of the extraction solution and small quantities of PVPP to avoid oxidation. The complete process was performed at 4°C. The mixture was centrifuged at 15000 g for 15 minutes and extracts of the samples (supernatant) were stored at -85°C until enzymatic quantification. In total were extracted seven samples per treatment.

The solutions for reaction of peroxidases (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) were adjusted from different previous protocols (Havir and McHale, 1987; Del Longo et al., 1993; Koshiha, 1993; Pandey and Agrawal, 1994) and are described in the Table 1. The preparing of reagents were calculated for a final volume of 200 μ L per sample, and quantifications were made in microplate reader. The content of proteins was determined by the Bradford method: the mixture of 5 μ L of extract, 15 μ L of water and 180 μ L of Bradford reagent had reads at 595 nm and the curve of protein was generated with 0, 2, 4, 6, 8 and 10 μ L of BSA 0.1%.

Table 1. Composition of solutions for enzymatic antioxidative reaction.

	POX	SOD	APX	CAT	GR
Buffer	Potassium phosphate 0.1M pH 6.8	Potassium phosphate 0.1M pH 7.8	Potassium phosphate 0.1M pH 6.0	Potassium phosphate 0.1M pH 6.8	Potassium phosphate 0.1M pH 7.5
Reagents	50 µL Buffer 50 µL Pyrogallol 80 mM 32 µL H ₂ O ₂ 125mM 61 µL H ₂ O	100 µL Buffer 15 µL NBT 1mM 52 µL Methionin 2.5 µL EDTA 10 mM 0.1 µL Riboflavin 15.4 µL H ₂ O	100 µL Buffer 16 µL Ascorbate 10 mM 17 µL H ₂ O ₂ 12.5 mM 60 µL H ₂ O	100 µL Buffer 20 µL H ₂ O ₂ 125mM 68 µL H ₂ O	10 µL Buffer 20 µL EDTA 10 mM 20 µL GSSG 10mM 40 µL MgCl ₂ 50 mM 118 µL H ₂ O 5 µL NADPH 4mM
Extract	7 µL	15 µL	7 µL	12 µL	7 µL
Reading	420 nm Interval: 4 s Duration: 1 min. Start with H ₂ O ₂ .	560 nm Punctual reading 1st reading after 30 minutes of dark. 2nd reading after 30 minutes of light.	290 nm, UV-plate Interval: 4 s Duration: 1 min. Start with H ₂ O ₂ .	240 nm, UV-plate Interval: 4 s Duration: 1 min. Start with H ₂ O ₂ .	340 nm Interval: 10 s Duration: 3 min. Start with extract.

Vigor and Tolerance

Five germinants from each treatment were sown in plastic nursery pots with sterilized sand. Once the first leaf emerged, seedlings were irrigated daily and each three days was added nutritive solution of Clark diluted 1:3. In the day 40 the dilution was increased to 1:2. Each liter of nutritive solution was prepared with 0,597 g Ca(NO₃)₂·4H₂O, 0,131 g KNO₃, 0,037 g KCl, 0,072 g NH₄NO₃, 0,148 g MgSO₄·7H₂O, 0,009 g Ca(H₂PO₄)₂·H₂O, 0,02 g MgCl₂·6H₂O, 1,4x10⁻³ g MnCl₂·4H₂O, 1,75x10⁻³ g H₃BO₃, 5,8X10⁻⁴ g ZnSO₄·7H₂O, 1x10⁻⁴ g (NH₄)₆Mo₇O₂₄·4H₂O, 1,2x10⁻⁴ g CuSO₄·5H₂O and 1,2x10⁻⁴ g Fe-EDTA.

At the end of 70 days, seedlings were measured, dried at 85°C for 48 hours and weighted.

The vigor index (VI) was calculated by the formula (Imran et al., 2015):

$$VI = SL * (\%G)$$

Where SL is the length of stem and %G is the germination.

The index of tolerance to As (TI), based in the approach of Reisinger et al. (2008), was calculated from the vigor as follows:

$$TI = VI(As_i) / VI(As_c) * 100$$

Where $VI(As_i)$ is the vigor index of the concentration i of As and $VI(As_c)$ is the vigor index of control seedlings, that means, without stratification nor As.

Statistical analysis

The differences between control and chilling imbibition were evaluated with Student's t-test and the effect of As was tested with one-way ANOVA. The post-hoc used was the Fisher's LSD. Correlations between morphological traits were performed to find some allometric pattern related with As concentration. All the statistical analysis were performed with the software Statistica v.10 (StatSoft Inc, 2011).

Results

The chilling imbibition increased the germination in more than 50% and that positive effect persisted even in presence of arsenic (As) (Figure 1). Only the treatment of 5.0 mg L^{-1} of As had a germination similar to that of the seeds imbibed at 25°C . The germination was delayed by As in seeds imbibed at 25°C , since the maximal germination with and without As was reached in the days 14 and 22, respectively. In the case of seeds imbibed at 3°C the germination dynamic was homogenized because practically at the day 18 all the treatments had reach the stabilization.

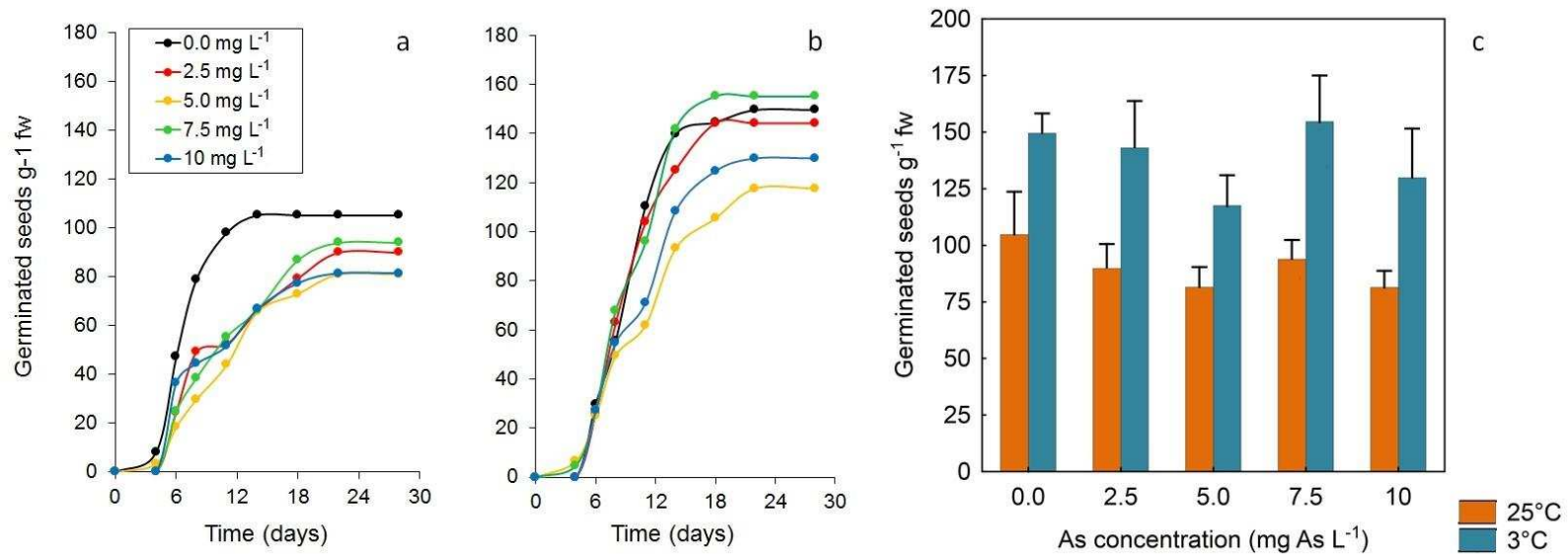


Figure 1. Effect of stratification and arsenic on germination of *Alnus acuminata*. Germinative dynamic of seeds pre-imbibed at 25°C (a) or at 3°C (b), and final germination after 30 days of treatment (c). A seed with radicle emergence was considered as germinated.

The early development was negatively affected by As when seeds were imbibed at 25°C (Figure 2). Despite that expansion of cotyledons overcame the 60% in the day 15, the presence of the first leaf was delayed and the survival in every concentration of As was below 40%; without As, germinants have a survival until the stage of seedling of 74%. When seeds were imbibed at 4°C, the emergency of cotyledons was delayed, but the final survival until seedling stage oscillated between 60% and 100%, being the best treatment the application of 7.5 mg L⁻¹As and the worst the application of 10 mg L⁻¹.

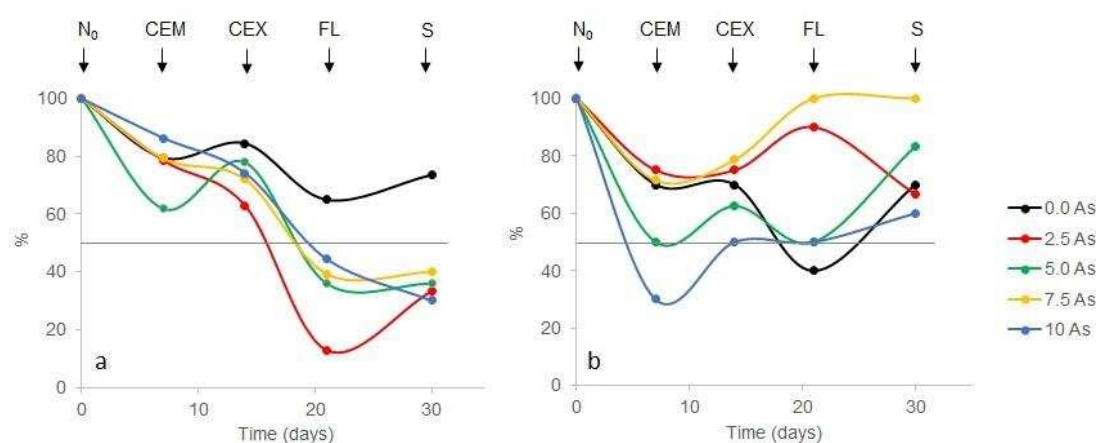


Figure 2. Early development of *A. acuminata* under 0, 2.5, 5.0, 7.5 and 10 mg L⁻¹ of As, germinated at 25°C imbibition (a) and 3°C imbibition (b). Values are percentages, from an initial number of germinated seeds (N₀), of reaching cotyledon emergence (CEM), cotyledon expansion (CEX), first leaf (FL) and survival (S). N₀ = 18-25.

Several biochemical and physiological traits of the germinants were different when comparing only the two types of imbibition without As. Seeds coming from chilling imbibition showed a lower entry of water, but gave germinants with higher fresh weight, higher content of aminoacids, glucose, and proteins. Also they presented a lower ratio of chlorophyll a/b and a lower content of starch than germinants coming from seeds imbibed at 25°C (Table 2).

Table 2. Effect of stratification (moist chilling at 3°C) in germination and physiology of germinated seeds. Asterisk denote significant differences with $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

		Temperature of Imbibition	
		25°C	3°C
Seeds	Imbibition (mg fw sem ⁻¹)	1.13±0.11	1.01±0.07**
	Leakage 20 h (µS g ⁻¹)	118.9±11.9	143.6±13.8**
	Germination (%)	6.94±1.73	10.5±2.67**
Seedlings 7-12 days	Axis length (mm)	11.27±1.09	11.75±1.35
	Fresh weight (mg)	2.29±0.53	2.81±0.58*
	Chlorophyll a (mg g ⁻¹ fw)	0.85±0.32	0.86±0.25
	Chlorophyll b (mg g ⁻¹ fw)	0.48±0.23	0.66±0.13
	Ratio chlorophyll a/b	2.43±0.71	1.32±0.36*
	Glucose (µmol g ⁻¹ fw)	1.21±0.27	2.26±0.75*
	Fructose (µmol g ⁻¹ fw)	1.06±0.42	1.28±0.33
	Sucrose (µmol g ⁻¹ fw)	10.12±2.03	9.14±1.95
	Aminoacids (µmol g ⁻¹ fw)	42.65±4.28	51.83±6.75*
	Starch (µmol g ⁻¹ fw)	10.41±0.9	5.28±1.6**
	Proteins (mg g ⁻¹ fw)	4.31±0.72	6.71±1.3**

The content of As in the germinants coming from chilling imbibition increased in logarithmic way in relation with the treatment of exposure to As. The highest concentration was of 67.6 µg g⁻¹ of dry weight in the treatment of 10 mg As L⁻¹. The only morphological variable that had a response in a similar pattern was the fresh weight, with a significant reduction from 0.0 to 7.5 mg L⁻¹ As, but the germinants of the treatment 10 mg L⁻¹ had a recovering of the biomass (Figure 3). In general, the absorption of As reduced the biomass and length of stem and root, but increased the diameter of cotyledons (Figure 3). Coefficients of determination of dry weight in function of total length were markedly higher in germinants with As than in the germinants without As (Figure 4).

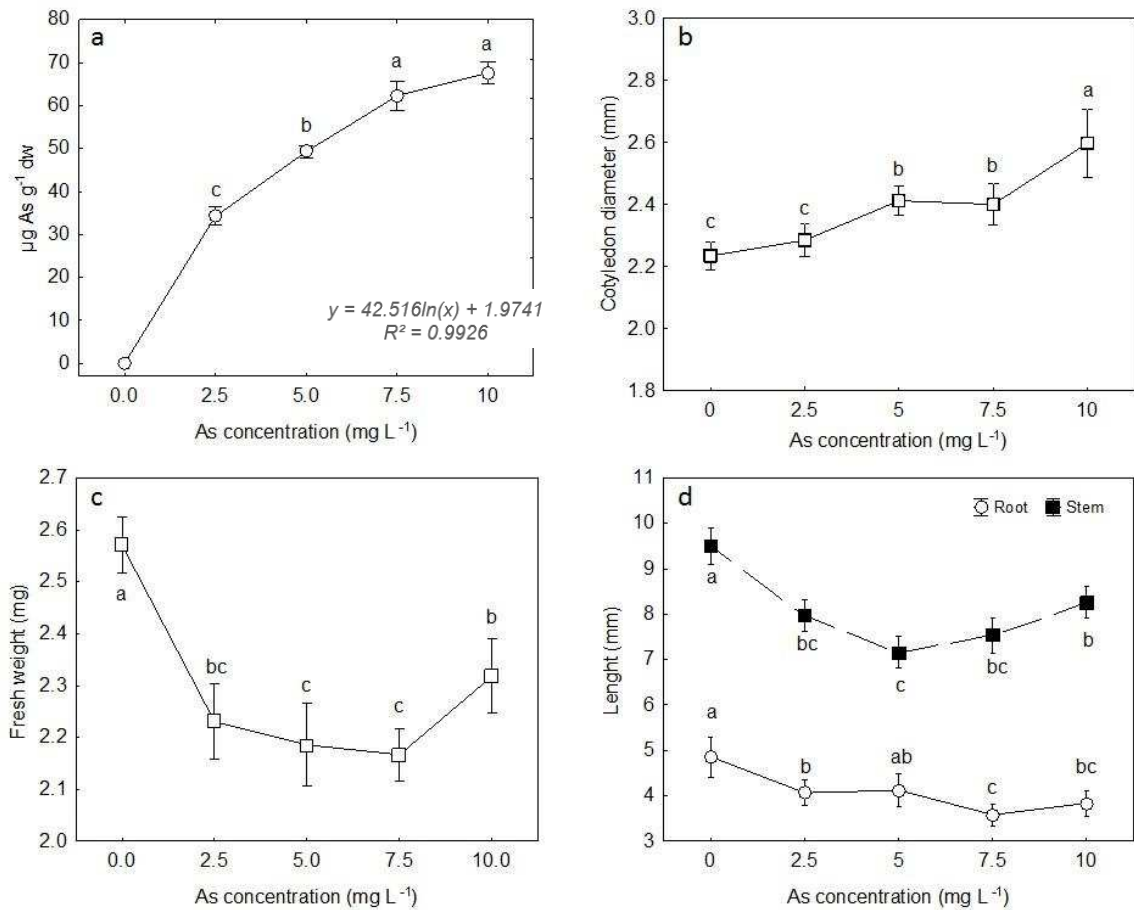


Figure 3. Accumulation of As (a) and growth (b, c, d) of germinants of 10-12 days. Seeds pre-treated with moist imbibition at 3°C were germinated under five concentrations of As. N = 5 (a), N = 15 (b, c, d).

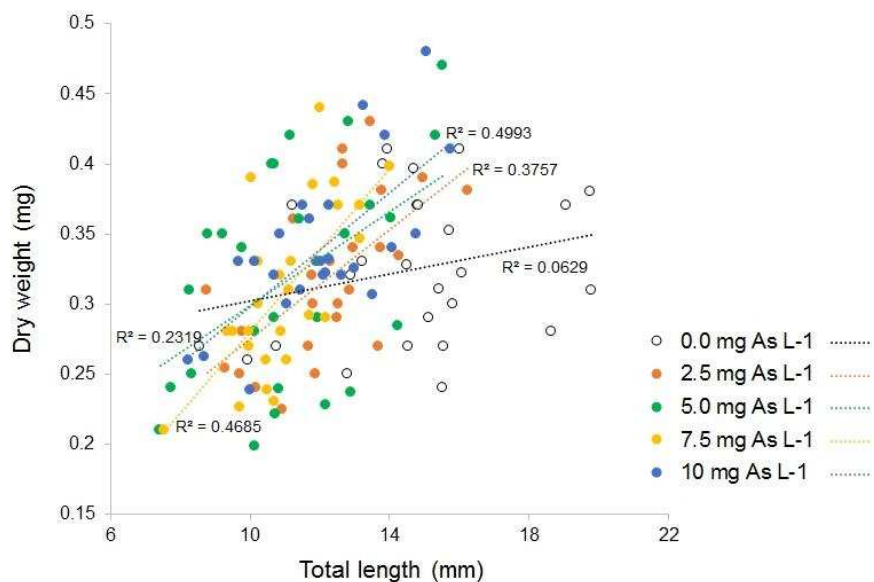


Figure 4. Allometric relationship between total length and dry biomass, in germinants coming from exposition to five concentrations of As.

There was not detected any emission of ethylene from pre-imbibed seeds, but in germinants the emission of ethylene from of 2.5 and 5.0 mg As L⁻¹ exceeded that from any other treatment. The germinants from treatments with 5.0, 7.5 and 10 mg As L⁻¹ had a higher content of starch, proteins and sucrose than germinants without As; also the treatment 10 mg As L⁻¹ showed a tendency of higher concentration of hexoses (Figure 5). The treatment of 7.5 mg L⁻¹ As showed the highest content of total chlorophylls but the lowest content of aminoacids.

The antioxidant enzymes POX and SOD showed the higher activity at the highest concentrations of As (7.5 mg L⁻¹ and 10 mg L⁻¹) during germination (Figure 6). The enzymes APX and CAT did not have any tendency associable to concentration of As, and the activity of GR diminished linearly with the increase of As. The treatment 7.5 mg As L⁻¹ showed a specific response of high activity of CAT and lower content of malate. In germinants without As the order of antioxidant activity was: CAT (10.4±1.4 μmol min⁻¹ g⁻¹ protein) > SOD (5.72±0.95 μmol min⁻¹ g⁻¹ protein) > POX (3.51±0.5 μmol min⁻¹ g⁻¹ protein) > APX (0.20±0.03 μmol min⁻¹ g⁻¹ protein) > GR (20.3±3.7 nmol min⁻¹ g⁻¹ protein).

Seedlings of 70 days from 3°C-preimbibition had a healthy phenotype, regardless the application of As during germination. In fact, in some traits as total dry weight and leaf dry weight the seedlings subjected to high concentrations of As showed better growth than seedlings from 25°C-preimbibition without As (Table 3). The seedlings from the treatment 3°C – 7.5 mg As L⁻¹ had the maximum means of leaf area, root length and dry weight, and join with the treatment 3°C – 10 mg As L⁻¹, the highest leaf dry weight. The stem length and number of leaves remained unalterable in relation to seed pretreatments or As concentration. In general, the chilling imbibition increased the vigor in more than 57% and increased the tolerance to As in 55%. In the imbibition at 25°C, the seeds with As had a vigor slightly lower than control seeds, only the treatment of 10 mg As L⁻¹ overcame the tolerance of 100%, but any of these differences were statistically significant.

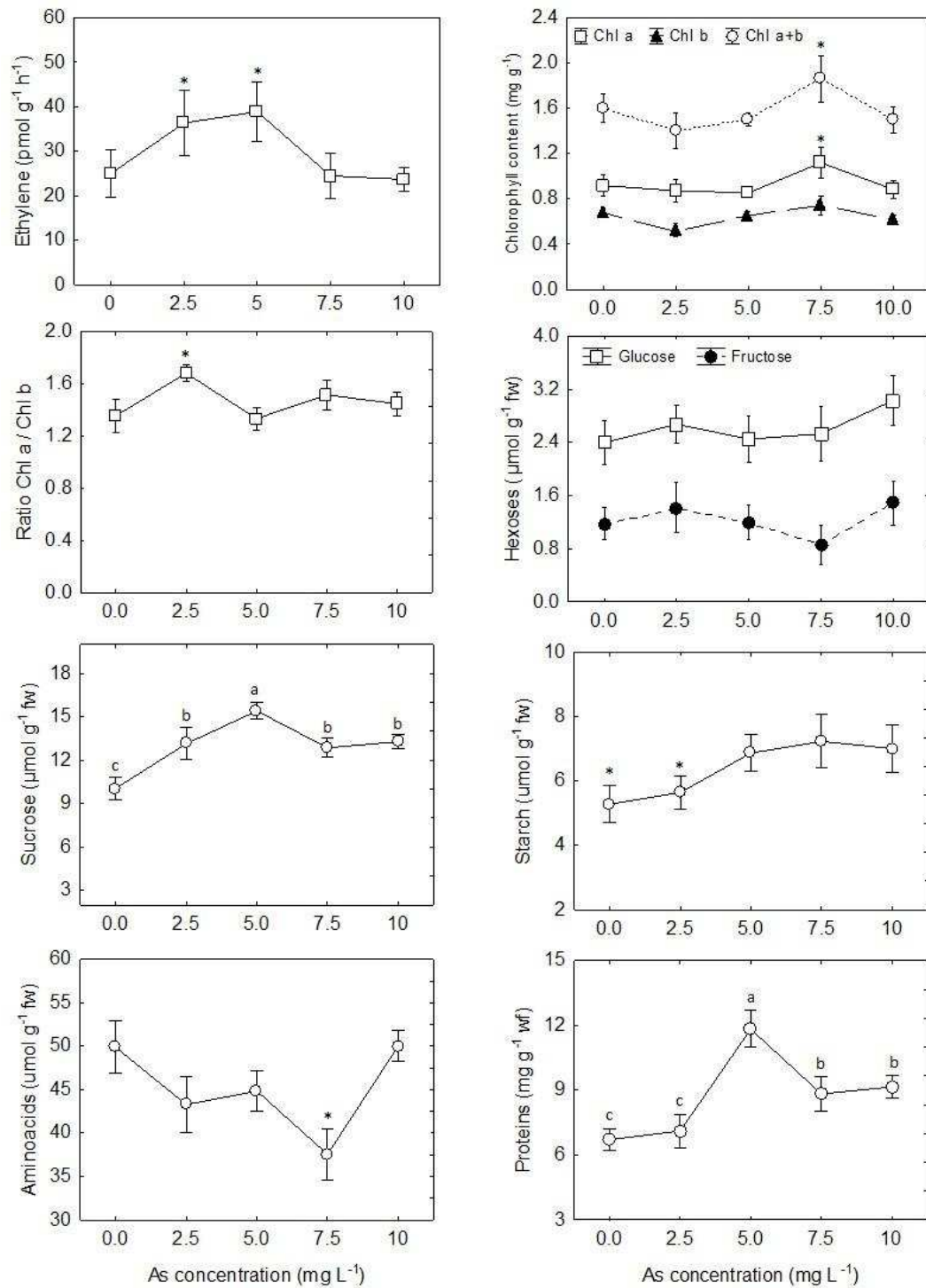


Figure 5. Ethylene production in germinants of 5 days (N=4) and content of chlorophylls, carbohydrates, aminoacids and proteins in germinants of 10-12 days (N=7), under five concentrations of As during germination. Asterisk denote a single treatment with significant differences (P<0.05). Letters denote many levels of significant differences (P<0.05).

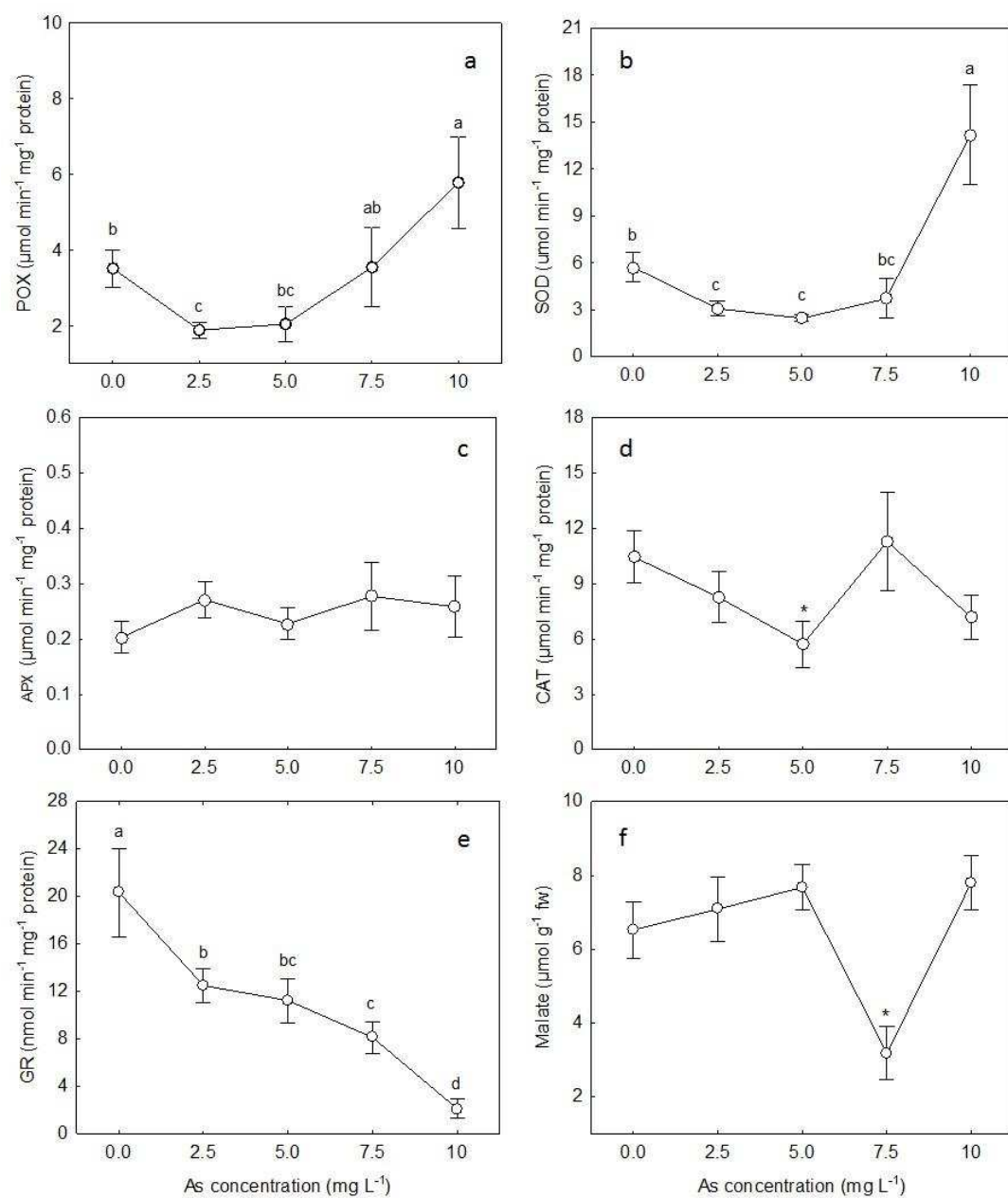


Figure 6. Responses of antioxidant enzymes (a- e) and malate (f) to five concentrations of As during the germination. N=8.

Table 3. Size, biomass, vigor and tolerance (mean±SD) of seedlings (70 days) coming from control (25°C) and stratified (3°C) seeds germinated under five concentrations of arsenic. Tolerance index was calculated from the vigor Index, and the seedlings 25°C-0.0As were used as reference of vigor. Asterisks denote a general, significant effect of chilling pretreatment with P≤0.05 (*) or P≤0.01 (**). Letters denote differences among As treatments inside the groups of imbibition, P≤0.05. N=5.

Seedling Trait	Pre-imbibition temperature	As concentration (mg L ⁻¹)				
		0.0	2.5	5.0	7.5	10
Leaf area (mm ²)	25°C	49.9±10	45±9 ^b	51±12.5	39.1±10.5 ^b	63.5±13.3 ^a
	3°C	47±11	48.3±7.1	58.5±13	61±16 ^a	58.9±3.9
Stem length (mm)	25°C	15.8±1.8	17.8±4.5	20±2.3	16.3±2.6	20.6±3.5
	3°C	17.9±2.1	18.8±2.5	21±3.8	17.2±3.6	19.2±2.8
Root length (mm)	25°C	28.5±2.6 ^b	26.4±2.8 ^b	28.8±5.1	33.1±3.8	33.6±11
	3°C	40.6±8.1 ^a	27.2±6 ^b	33.6±8.2	40.7±10.2 ^a	36±6.6
Stem/root ratio	25°C	0.56±0.08	0.69±0.2	0.72±0.18	0.54±0.08	0.69±0.15
	3°C	0.47±0.1 ^b	0.77±0.19 ^a	0.64±0.13	0.52±0.17	0.58±0.11
Number of leaves	25°C	4.6±0.6	4.8±0.8	4.6±1	4.5±0.8	4.8±0.9
	3°C	4.8±0.8	4.4±0.5	4.8±0.8	5.2±1.1	5±0.7
Total dry weight (mg)	25°C	2.03±0.4 ^b	2.09±0.5	2.52±0.6	2.12±0.3 ^b	3.07±0.6 ^a
	3°C	2.45±0.6	2.32±0.3	2.6±0.3	2.99±0.5 ^a	2.81±0.4 ^a
Leaf dry weight (mg)	25°C	0.78±0.18 ^b	0.79±0.17	0.92±0.3	0.54±0.12 ^b	1.19±0.23 ^a
	3°C	0.84±0.22	0.79±0.12	0.85±0.26	1.09±0.32 ^a	1.12±0.18 ^a
Vigor Index	25°C	127.4±25	123.1±29	125.4±27	117.7±21	129.1±23
	3°C ^{**}	205.9±24	206.9±39	180±29	204.7±33	191.8±44
Tolerance Index	25°C	100	96.7±22.9	98.5±21.2	92.4±16.7	101.4±18.2
	3°C*		162.5±32	133.5±24.4	160.8±27.2	150.6±31.5

Discussion

The results of this study demonstrated that seeds of *Alnus acuminata* pretreated with cold stratification are able to germinate under the influence of up 10 mg As L⁻¹, and seedlings coming from these seeds growth totally healthy. Furthermore, the prechilling imbibition at 3°C enhances the vigor, and this effect was unaltered by the presence of As.

Imbibition at chilling temperatures slow down the hydration of seeds because the viscosity of water at 3°C, avoiding the anoxic conditions and allowing the start of metabolic reactions in the embryo during the germination (Chakraborty and Kar, 2008). The molecular and biochemical mechanisms behind the effect of cold stratification are currently controversial, but there are evidences that genes activated by low temperatures are related with synthesis of gibberellins (Nonogaki, 2006; Staszak et al., 2017) and chilling imbibition increases the production of ROS that promote the germination (Dębska et al., 2013; Su et al., 2016). The positive effect of stratification on germination was already demonstrated in *Alnus glutinosa*, *A. rugosa*, *A. crispa* and *A. viridis* (Baskin and Baskin, 2001; De Atrip and O'Reilly, 2007; Gosling et al., 2009).

The chilling imbibition improved the early development when seeds were exposed to As, and the positive effect was evident specifically in the pass from germinant to seedling, that is, in the apparition of the first leaf. In consequence, the survival until the seedling stage of more than two leaves in a sterile substrate overcame 50%. It is evident that some genetic mechanism are involved in this regulation and deserve a deeper research. In fact, if survival of germinants were included in the calculation of vigor, the effect of stratification would be overwhelming in relation with preimbibition at room temperature. The biochemical analysis showed that germinants from pre-chilled treatment active more quickly their metabolism, by degradation of starch and increase of concentration of glucose that in turn is oxidized for synthesis of aminoacids and proteins (Table 2).

The accumulation of As in germinants of *A. acuminata* is perhaps the best evidence of its tolerance. This accumulation at least duplicated the content of As in seedlings of rice, tobacco or lettuce that suffered a strong reduction in germination, nutrition, growth and yield at similar concentrations of applied As (Pavlík et al., 2010; Talano et al., 2014; Zia et al., 2017). In this study, the maximum reduction was of 28% in the root length at 7.5 mg As L⁻¹ or 21% in the stem length at 5.0 mg As L⁻¹, but these impairing is recovering when germinants growth until the stage of seedling (Figure 3, Table 3). In the same way, the maximal concentration of As only diminished the stem length of germinants in 15% or the fresh weight in 10%, therefore there is not a linear negative correspondence between growth and accumulation of As. The main allometric pattern found was a linear increment of biomass as a function of the stem length in germinants from arsenic treatments and that was not evident in germinants

without arsenic, which suggest an optimization of growth in presence of As. The linear increase of size of the cotyledons seems to be a key piece in the tolerance, because this part of the germinants could be used for As sequestration.

The explaining of the observed growth depression in germinants influenced by As followed by a recovering in the stage of seedlings, could involve at least two different mechanisms of tolerance. The first fact is the emission of ethylene because of the As treatment, specifically at 2.5 and 5.0 mg As L⁻¹. There are evidences that ethylene is perhaps the first hormone to signalize stress by heavy metals, since pollutants as Cu, Cr and Cd interfere in the transcription of precursors in the biosynthesis of ethylene, which in turns regulates the responses to stress (Keunen et al., 2016; Bucker-Neto et al., 2017). The inhibition of elongation is one of these responses (Sun et al., 2009). In the other side, the null detection in imbibed seeds demonstrated that ethylene is not associated to breaking of dormancy of *A. acuminata*.

Earlier research describes increase of proteins (Mishra and Dubey, 2006; Li et al., 2007) and decrease of carbohydrates (Kaur et al., 2012) and chlorophylls (Li et al., 2007; Mahdih et al., 2013; Upadhyaya et al., 2014) as biochemical effects of arsenic toxicity in seedlings. The responses of aminoacids are variable (Mishra and Dubey, 2006; Pavlík et al., 2010). In germinants of *A. acuminata* from arsenic treatments, the content of proteins, starch, sucrose and even chlorophyll a (at 7.5 mg As L⁻¹) increased. Particularly, sucrose have protectant and antioxidant functions in stressing conditions (Keunen et al., 2013) and probably oligosaccharides as raffinose or stachyose are hydrolyzed to maintain high concentrations of sucrose and hexoses without starch depletion (Bialecka and Kępczyński, 2007; Gangl and Tenhaken, 2016).

The antioxidant activity is another strategy line of tolerance to As in *A. acuminata*. The ROS scavenging was in charge of two enzymes: SOD and POX. The SOD triplicated its activity from 7.5 mg As L⁻¹ to 10 mg As L⁻¹ and neutralize the superoxide radical by conversion into hydrogen peroxide. Later, the POX converts hydrogen peroxide into water. A third enzyme, the GR decreased with the increase of As, what suggest a depletion of glutathione due to synthesis of phytochelatin for As complexation and sequestration (Mishra et al., 2008; Song et al., 2010; Yadav, 2010). It is important to highlight that the reaction of enzymes was centered basically in the maximum application, of 10 mg L⁻¹ As. Apparently, at lower concentrations the response or the antioxidant machinery is barely actioned. In *Myracrodruon urudeuva*, a tree species that growth in soils contaminated by heavy metals, the germination under

10 mg L⁻¹ of As was reduced in 35% and the increase of antioxidant activity was of 100% in SOD, 325% in CAT and 20% in GR. The activity of APX was invariant (Gomes et al., 2013). In seedlings of rice the principal enzymes that deal with the oxidative stress caused by arsenic are POX and SOD. CAT activity was negligible (Li et al., 2007).

From all the treatments of As tested, the concentration of 7.5 mg As L⁻¹ was the most particular, because to trigger the increase of chlorophyll content and CAT activity and the reduction of malate concentration. These biochemical responses could be related with the fact that growth of seedlings was optimized until the point to maximize the vigor and tolerance. The behavior completely antagonist of CAT and malate (Figure 6, d and f) could be an evidence of the exclusion of As complexed with malate or derived organic acids through an anion efflux channel in the plasma membrane (Inostroza-Blancheteau et al., 2012) and the malate-dependent activation of catalase, mediated by the valve enzyme NADP-malate dehydrogenase (Heyno et al., 2014).

Conclusions

A chilling pretreatment on seeds of *Alnus acuminata* enhances the germination tolerance to arsenic (As), at least up 10 mg As L⁻¹. The chilling pretreatment accelerate the metabolism, increasing the concentration of glucose, aminoacids and proteins in germinants of 12-15 days. The effect of As in germinants coming from pretreated seeds can be summarized in increase of proteins, starch and sucrose, without negative effects in chlorophylls. Also, these germinants present an allometrical pattern of increase of biomass per unity of size that is not demonstrated in germinants without arsenic. The increase of size of cotyledons suggest that this structure could be used for As sequestration at quantities that are toxic in the most cases. The antioxidant activity of SOD, POX and probably phytochelatins would be involved in the tolerance of germinants to As, allowing their growth until healthy seedlings of 70 days. Finally, it was demonstrated that chilling treatment improves substantially the vigor of seeds, even at high concentrations of As.

References

- Adriano, D.C., 2001. Trace elements in terrestrial environments: Biochemistry, Bioavailability and risks of metals, 2nd ed. Springer-Verlag, New York.
- Anandarajah, K., Kott, L., Beversdorf, W.D., McKersie, B.D., 1991. Induction of desiccation tolerance in microspore-derived embryos of *Brassica napus* L. by thermal stress. *Plant Sci.* 77, 119-123.
- Baskin, C.C., Baskin, J.M., 2001. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. Academic Press, San Diego, USA.
- Białecka, B., Kępczyński, J., 2007. Changes in concentrations of soluble carbohydrates during germination of *Amaranthus caudatus* L. seeds in relation to ethylene, gibberellin A3 and methyl jasmonate. *Plant Growth Regul.* 51, 21-31.
- Bothe, H., 2011. Plants in heavy metal soils, in: Sherameti, I., Varma, A. (Eds.), *Detoxification of Heavy Metals*. Springer-Verlag, Berlin, pp. 35-57.
- Bourgoin, A., Simpson, J.D., 2004. Soaking, moist-chilling, and temperature effects on germination of *Acer pensylvanicum* seeds. *Can. J. Forest Res.* 34, 2181-2185.
- Bücker-Neto, L., Paiva, A.L.S., Machado, R.D., Arenhart, R.A., Margis-Pinheiro, M., 2017. Interactions between plant hormones and heavy metals responses. *Genet. Mol. Biol.* 40, 373-386.
- Bundschuh, J., Giménez-Forcada, E., Guerequiz, R., Pérez-Carrera, A., Garcia, M.E., Mello, J., Deschamps, E., 2008. Fuentes geogénicas de arsénico y su liberación al medio ambiente, in: Bundschuh, J., Pérez-Carrera, A., Litter, M. (Eds.), *Distribución del Arsénico en las Regiones Iberica e Iberoamericana*. Editorial Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo, Buenos Aires, pp. 33-47.
- Cross, J.M., von Korff, M., Altmann, T., Bartzetko, L., Sulpice, R., Gibon, Y., Palacios, N., Stitt, M., 2006. Variation of enzyme activities and metabolite levels in 24 *Arabidopsis* accessions growing in carbon-limited conditions. *Plant Physiol.* 142, 1574-1588.
- Chakraborty, R., Kar, R., 2008. Differential water uptake kinetics in axes and cotyledons during seed germination of *Vigna radiata* under chilling temperature and cycloheximide treatment. *Braz. J. Plant Phys.* 20, 277-284.

- Chao, D.-Y., Chen, Y., Chen, J., Shi, S., Chen, Z., Wang, C., Danku, J.M., Zhao, F.-J., Salt, D.E., 2014. Genome-wide association mapping identifies a new arsenate reductase enzyme critical for limiting arsenic accumulation in plants. *PLOS Biol.* 12, e1002009.
- De Atrip, N., O'Reilly, C., 2007. Germination response of alder and birch seeds to applied gibberellic acid and priming treatments in combination with chilling. *Ann. Forest Sci.* 64, 385-394.
- Dębska, K., Krasuska, U., Budnicka, K., Bogatek, R., Gniazdowska, A., 2013. Dormancy removal of apple seeds by cold stratification is associated with fluctuation in H₂O₂, NO production and protein carbonylation level. *J. Plant Physiol.* 170, 480-488.
- Del-Toro-Sánchez, C.L., Zurita, F., Gutiérrez-Lomelí, M., Solis-Sánchez, B., LauraWence-Chávez, L., Rodríguez-Sahagún, A., Castellanos-Hernández, O.A., Vázquez-Armenta, G., Siller-López, F., 2013. Modulation of antioxidant defense system after long term arsenic exposure in *Zantedeschia aethiopica* and *Anemopsis californica* *Ecotox. Environ. Safe.* 94, 67-72.
- Del Longo, O.T., González, C.A., Pastori, G.M., Trippi, V.S., 1993. Antioxidant defenses under hyperoxygenic and hyperosmotic conditions in leaves of two lines of maize with differential sensitivity to drought. *Plant Cell Physiol.* 34, 1023-1028.
- Duarte, D.M., Gomes, M.P., Barreto, L.C., Matheus, M.T., Garcia, Q.S., 2012. Toxic trace elements effects on seed germination of four Brazilian Savanna tree species. *Seed Sci. Technol.* 40, 425-432.
- Felipe, R.T.A., Leão, G.A., Oliveira, J.A., 2009. Crescimento e teores de arsênio em plântulas de *Leucaena leucocephala* e *Sesbania grandiflora*. *Ceres* 56, 219-224.
- Fernie, A.R., Roscher, A., Ratcliffe, R.G., Kruger, N.J., 2001. Fructose 2, 6-bisphosphate activates pyrophosphate: fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. *Planta* 212, 250-263.
- Finnegan, P.M., Chen, W., 2012. Arsenic toxicity: the effects on plant metabolism. *Front. Physiol.* 3, 182.

- Gangl, R., Tenhaken, R., 2016. Raffinose family oligosaccharides act as galactose stores in seeds and are required for rapid germination of *Arabidopsis* in the dark. *Front. Plant Sci.* 7, 1115.
- Gomes, M., Carneiro, M., Nogueira, C., Soares, A., Garcia, Q., 2013. The system modulating ROS content in germinating seeds of two Brazilian savanna tree species exposed to As and Zn. *Acta Physiol. Plant.* 35, 1011-1022.
- Gosling, P.G., McCartan, S.A., Peace, A.J., 2009. Seed dormancy and germination characteristics of common alder (*Alnus glutinosa* L.) indicate some potential to adapt to climate change in Britain. *Forestry* 82, 573-582.
- Havir, E.A., McHale, N.A., 1987. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiol.* 84, 450-455.
- Heyno, E., Innocenti, G., Lemaire, S.D., Issakidis-Bourguet, E., Krieger-Liszkay, A., 2014. Putative role of the malate valve enzyme NADP-malate dehydrogenase in H₂O₂ signalling in *Arabidopsis*. *Philos. T. Roy. Soc. B.* 369, 20130228.
- Houšková, K., Martiník, A., 2015. Does prolonged stratification of Douglas fir influence the yield of seedlings? *J. Forest Sci.* 61, 268-273.
- Imran, M.A., Sajid, Z.A., Chaudhry, M.N., 2015. Arsenic (As) toxicity to germination and vegetative growth of sunflower (*Helianthus annuus* L.). *Pol. J. Environ. Stud.* 24, 1993-2002.
- Inostroza-Blancheteau, C., Rengel, Z., Alberdi, M., de la Luz Mora, M., Aquea, F., Arce-Johnson, P., Reyes-Díaz, M., 2012. Molecular and physiological strategies to increase aluminum resistance in plants. *Mol. Biol. Rep.* 39, 2069-2079.
- Isayenkov, S.V., Maathuis, F.J.M., 2008. The *Arabidopsis thaliana* aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. *FEBS Lett.* 582, 1625-1628.
- Kaur, S., Singh, H.P., Batish, D.R., Negi, A., Mahajan, P., Rana, S., Kohli, R.K., 2012. Arsenic (As) Inhibits radicle emergence and elongation in *Phaseolus aureus* by altering starch-metabolizing enzymes vis-à-vis disruption of oxidative metabolism. *Biol. Trace Elem. Res.* 146, 360-368.
- Keunen, E., Peshev, D., Vangronsveld, J., Van Den Ende, W., Cuypers, A., 2013. Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. *Plant Cell Environ.* 36(7), 1242-1255.
- Keunen, E., Schellingen, K., Vangronsveld, J., Cuypers, A., 2016. Ethylene and metal stress: small molecule, big impact. *Front. Plant Sci.* 7, 23.

- Koshiha, T., 1993. Cytosolic ascorbate peroxidase in seedlings and leaves of maize (*Zea mays*). *Plant Cell Physiol.* 34(5), 713-721.
- Li, C.X., Feng, S.L., Shao, Y., Jiang, L.N., Lu, X.Y., Hou, X.L., 2007. Effects of arsenic on seed germination and physiological activities of wheat seedlings. *J. Environ. Sci.* 19, 725–732.
- Lin, J., Shi, Y., Tao, S., Yu, X., Yu, D., Yan, X., 2017. Seed-germination response of *Leymus chinensis* to cold stratification in a range of temperatures, light and low water potentials under salt and drought stresses. *Crop Pasture Sci.* 68, 188-194.
- Mahdieh, S., Ghaderian, S.M., Karimi, N., 2013. Effect of arsenic on germination, photosynthesis and growth parameters of two winter wheat varieties in Iran. *J. Plant Nutr.* 36, 651-664.
- Mishra, S., Dubey, R.S., 2006. Inhibition of ribonuclease and protease activities in arsenic exposed rice seedlings: Role of proline as enzyme protectant. *J. Plant Physiol.* 163, 927-936.
- Mishra, S., Srivastava, S., Tripathi, R.D., Trivedi, P.K., 2008. Thiol metabolism and antioxidant systems complement each other during arsenate detoxification in *Ceratophyllum demersum* L. *Aquat. Toxicol.* 86, 205-215.
- Moreno-Jiménez, E., E.Esteban, R.O.Carpaena-Ruiz, J.M.Peñalosa, 2009. Arsenic-and mercury-induced phytotoxicity in the Mediterranean shrubs *Pistacia lentiscus* and *Tamarix gallica* grown in hydroponic culture. *Ecotox. Environ. Safe.* 72, 1781-1789.
- Nonogaki, H., 2006. Seed germination—the biochemical and molecular mechanisms. *Breeding Sci.* 56, 93-105.
- Nunes-Nesi, A., Carrari, F., Gibon, Y., Sulpice, R., Lytovchenko, A., Fisahn, J., Graham, J., Ratcliffe, R.G., Sweetlove, L.J., Fernie, A.R., 2007. Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. *Plant J.* 50, 1093-1106.
- Pandey, J., Agrawal, M., 1994. Evaluation of air pollution phytotoxicity in a seasonally dry tropical urban environment using three woody perennials. *New Phytol.* 126, 53-61.
- Pavlík, M., Pavlíková, D., Staszková, L., Neuberg, M., Kaliszová, R., Száková, J., Tlustoš, P., 2010. The effect of arsenic contamination on amino acids metabolism in *Spinacia oleracea* L. *Ecotox. Environ. Safe.* 73(6), 1309-1313.

- Porra, R., Thompson, W., Kriedemann, P., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *BBA-Bioenergetics* 975, 384-394.
- Rawat, B.S., Khanduri, V.P., Sharma, C.M., 2008. Beneficial effects of cold-moist stratification on seed germination behaviors of *Abies pindrow* and *Picea smithiana*. *J. Forestry Res.* 19, 125-130.
- Reisinger, S., Schiavon, M., Terry, N., Pilon-Smits, E.A., 2008. Heavy metal tolerance and accumulation in Indian mustard (*Brassica juncea* L.) expressing bacterial γ -glutamylcysteine synthetase or glutathione synthetase. *Int. J. Phytoremediat.* 10, 440-454.
- Sanal, F., Seren, G., Güner, U., 2014. Effects of arsenate and arsenite on germination and some physiological attributes of barley *Hordeum vulgare* L. *B. Environ Contam. Tox.* 92, 483–489.
- Shahi-Gharahlar, A., Yavari, A.R., Khayyat, M., Jalali, N., Farhoudi, R., 2012. Effects of soaking temperature, stratification, potassium nitrate and gibberellic acid on seed germination of loquat trees. *J. Plant Nutr.* 35(11), 1735-1746.
- Song, W.Y., Park, J., Mendoza-Cózatl, D.G., Suter-Grotemeyer, M., Shim, D., Hörstensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J., Lee, Y., Martinoia, E., 2010. Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *P. Natl. Acad. Sci. USA.* 7, 21187–21192.
- Soon, Y.K., 1998. Determination of arsenic and mercury in plant tissue, in: Kalra, Y.P. (Ed.) *Handbook of Reference Methods for Plant Analysis: Tissue Tests Let Plants Speak*. CRC Press, Boca Raton USA, pp. 183-192.
- Srivastava, S., Akkarakan, J.J., Supasanna, P., D'Souza, S.F., 2013. Response of adenine and pyridine metabolism during germination and early seedling growth under arsenic stress in *Brassica juncea*. *Acta Physiologiae Plantarum* 35, 1081-1091.
- Staszak, A.M., Guzicka, M., Pawłowski, T.A., 2017. Signalling regulators of abscisic and gibberellic acid pathways are involved in dormancy breaking of Norway maple (*Acer platanoides* L.) seeds. *Acta Physiol. Plant.* 39, 251.

- Su, L., Lan, Q., Pritchard, H.W., Xue, H., Wang, X., 2016. Reactive oxygen species induced by cold stratification promote germination of *Hedysarum scoparium* seeds. *Plant Physiol. Biochem.* 109, 406-415.
- Sun, P., Tian, Q.-Y., Chen, J., Zhang, W.-H., 2009. Aluminium-induced inhibition of root elongation in *Arabidopsis* is mediated by ethylene and auxin. *J. Exp. Bot.* 61, 347-356.
- Talano, M.A., Oller, A.L.W., González, P., González, S.O., Agostini, E., 2014. Effects of arsenate on tobacco hairy root and seedling growth, and its removal. *In Vitro Cell. Dev. Biol. Plant* 50, 217-225.
- Talukdar, D., 2011. Effect of arsenic-induced toxicity on morphological traits of *Trigonella foenum-graecum* L. and *Lathyrus sativus* L. during germination and early seedling growth. *Curr. Res. J. Biol. Sci.* 3, 116-123.
- Upadhyaya, H., Shome, S., Roy, D., Bhattacharya, M.K., 2014. Arsenic induced changes in growth and physiological responses in *Vigna radiata* seedling: effect of curcumin interaction. *Am. J. of Plant Sci.* 5, 3609.
- Weitbrecht, K., Müller, K., Leubner-Metzger, G., 2011. First off the mark: early seed germination. *J. Exp. Bot.* 62, 3289-3309.
- Yadav, S.K., 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *S. Afr. J. Bot.* 76, 167-179.
- Zhao, F., Ma, J., Meharg, A., McGrath, S., 2009. Arsenic uptake and metabolism in plants. *New Phytol.* 181, 777-794.
- Zia, Z., Bakhat, H.F., Saqib, Z.A., Shah, G.M., Fahad, S., Ashraf, M.R., Hammad, H.M., Naseem, W., Shahid, M., 2017. Effect of water management and silicon on germination, growth, phosphorus and arsenic uptake in rice. *Ecotox. Environ. Safe.* 144, 11-18.

APPENDICES

1. Germinants of 7-12 days coming from seeds imbibed at 25°C (A) and 3°C (B)



2. Seedlings of 70 days coming from seeds imbibed at 25°C (A) and 3°C (B).



CONCLUSÕES GERAIS

Nos solos pós-mina de Samacá (Boyacá-Colombia) não existe uma alta concentração de metais pesados, mas uma alta acidificação, alta saturação de alumínio e alta concentração de arsênio são características químicas que geram limitações para o crescimento vegetal. Foi possível diferenciar as espécies susceptíveis das espécies potencialmente tolerantes, e uma dessas espécies, *Alnus acuminata*, foi a única árvore que tem distribuição exclusiva em rejeitos de carvão.

A germinação de *A. acuminata* é por natureza altamente variável. Porém, as melhores condições germinativas que maximizam a produção de plântulas são: Estratificação húmida a 4°C por um tempo inferior a 7 dias, semeadura em papel germitest, condições em câmara aclimatizada de fotoperíodo 12/12 h e temperatura alterna 20-30°C. As condições de luz não favorecem a germinação per se mas sim são necessárias para um ótimo desenvolvimento dos germinados até estado de plântula com folhas verdadeiras.

Além dos efeitos positivos na quebra de dormência, a estratificação húmida acelera o metabolismo em sementes germinadas: síntese de açúcares simples a partir de amido, síntese de proteínas e alta concentração de aminoácidos e clorofilas. Quando as sementes são germinadas em concentrações de arsênio similares às encontradas em solos de Samacá, foi evidente uma menor sobrevivência até o estado de plântula com folhas verdadeiras. Porém, um pretratamento de estratificação húmida inclusive aumenta a sobrevivência em comparação com sementes que nunca foram submetidas ao arsênio. Mesmo com uma acumulação de arsênio em sementes germinadas de quase 70 $\mu\text{g g}^{-1}$, a estratificação húmida melhorou a tolerância e o vigor analisado em plântulas de 70 dias.

Todos os resultados obtidos nesta pesquisa facilitam a produção de plântulas que possam ser testadas em campo para estabelecer se *Alnus acuminata* é a espécie nativa que pode ser usada para recuperação de solos pós-mina em Samacá.