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ORIGINAL ARTICLE

Effects of ammonium salts on oleaster (*Elaeagnus angustifolia*)

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Abstract *– Oleaster (Russian olive, Elaeagnus angustifolia) trees are highly tolerant against a variety of abiotic stresses (water, temperature, salt, and other chemicals). Therefore, they can be used for rehabilitation of contaminated and/or low quality soils (brownfields, dump sites, wastelands, etc.). In order to study responses of oleaster to environmental stress in vivo and in vitro, we successfully sterilized and initiated its callus cultures, regenerated shoots and roots and finally whole plants from the callus. Application of ammonium (in the form of sulfate salt) to the regenerated plantlets at concentrations higher than 10 mg* L^{-1} *inhibited root growth, reduced the leaf chlorophyll content and the activity of the enzyme glutamate dehydrogenase. At the same time, it induced activities of the stress marker enzyme glutathione S-transferase in the root and shoot tissues of the plant.*

Keywords *- Elaeagnus angustifolia, ammonia, stress, micropropagation*

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Introduction

Due to environmental pollution and extensive agricultural use the area of contaminated and low fertility soils is continuously enlarging worldwide. Recultivation of such soils is highly important for sustainable agriculture. Planting and cultivation of stress tolerant plants with the ability of taking up chemical pollutants and symbiotic aerial N_2 fixation is a highly efficient approach to improve soil fertility with limitations as regards to heavy metal uptake [1].

Elaeagnus species are saline and alkaline tolerant plants that grow well on disturbed and polluted soils and can be cultivated under a wide range of climatic conditions. Therefore, they can be used for land recultivation and windbreaks [1]. Oleaster (*Elaeagnus angustifolia*) is a large, spiny shrub often growing as a small tree. It is native to Eurasia but today it can be found in many regions of the world. As a highly stress tolerant plant it can rapidly colonize new areas and crowd out or replace native species. Therefore, it is often considered as an invasive species or weed. Indeed, the control of the spread of oleaster is a challenge. Interestingly, the propagation of oleaster is not simple either, because the germination rate of its seeds is very low even after months of cold-treatment. On the other hand, vegetative propagation *in vivo* or even *in vitro* is quite straightforward [2] [3].

Elaeagnus species are able to grow in symbiosis with the N_2-fixing bacteria *Frankia*, which use prokaryotic NifH, nitrogenase reductase enzymes [4] (Figure 1). This symbiosis results in nitrogen fixing nodules developing on the roots. Due to this symbiotic association, the growth of oleaster is independent from the soluble nitrogen nutrient content of the soil.

The genus *Frankia* was described first as filamentous fungus and named by J. Brunchorst in 1886 to honor the biologist A. B. Frank, and only in 1970 was classified to prokaryotic Actinobacteria (syn.: Actinomycetales). Complete circular genome sequences of *Frankia* strains are available with size range from 5,433,628 bp (NCBI # CP000249.1) to 8,982,042 bp (NCBI # CP000820), with very high GC ratio (71.2 %), low gene (7,377) and protein numbers (7,191) (NCBI # NC_009921.1).

In total, three main *Frankia* strains were isolated from species of three plant orders of Fagales (all *Alnus* species, some species of *Casuarina, Allocasuarina, Coriaria, Morella, Myrica, Gymnostoma*), two genera of Cucurbitales (*Coriaria and Datisca*), and numerous genera of Rosales (*Elaeagnus, Hippophae, Shepherdia, Colletia, Discaria, Kentrothamnus, Retanilla, Trevoa, Caenothus, Dryas, Purshia, Chamaebatia* and *Cercocarpus*). The 16S ribosomal DNA sequences of the root nodules of these species indicated that the nodules are formed by *Frankia* [5].

Starting in the middle of the last century the "green revolution" was based on the excessive use of mineral N-fertilizers that lead to volatilization of ammonia (NH_3, a greenhouse gas) and the deposition of ammonium ions (NH_4^+) in soils [6]. Soil solutions derived from agricultural areas may reach ammonium levels as high as 40 mM [7], while forest-floor soil solutions and landfill leachates contain an order of magnitude less [8], [9]. Although ammonia is the final form of inorganic nitrogen prior to the biosynthesis of organic nitrogen compounds [10], paradoxically at higher concentrations it is phytotoxic [11] that may result in limitations of the yield. Symptoms of ammonium toxicity most often include ammonium hyperaccumulation in tissues [12]–[14] and are coupled with

a disruption in cation homeostasis, leaf chlorosis, root growth inhibition, and reduced plant biomass. Ammonium sensitivity can also be observed in animals and humans [9].

An ammonium detoxification pathway is the reversible reaction of ammonia with 2-oxoglutaric acid to synthesize glutamic acid catalyzed by glutamate dehydrogenase (GDH; EC 1.4.1.2): this route is activated by ammonia concentrations above normal levels [15].

Materials and methods

Plant material

In vitro culture and micropropagation of oleaster was carried out as described previously [17]. Briefly, small branches of oleaster were collected from a tree, cut to 20 mm long pieces, and surface sterilized. First calli were initiated, subsequently whole plants were regenerated.

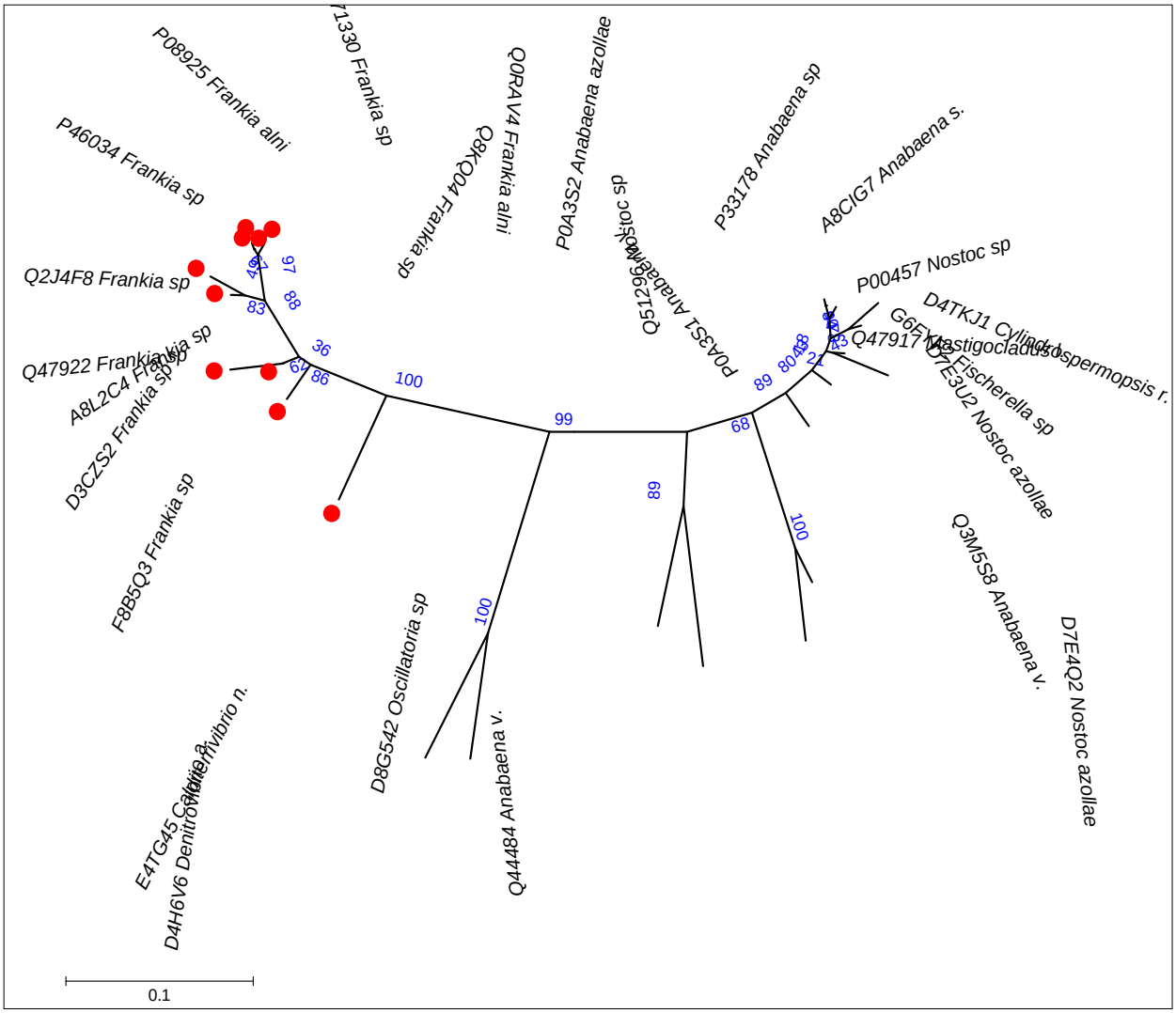

Figure 1. *Phylogeny of prokaryotic NIFH (NITROGENASE REDUCTASE Fe-S) enzymes including Frankia accessions. Sequences were downloaded from UniProt server. Sequence alignments (350 aa) and ML (Maximum Likelihood) dendrogram was edited by MEGA4 program. Accession numbers, high boot strap values (1000 replicates), and aa-changes per site (scale 0.1) are indicated. Red dots indicate the Frankia accessions.*

Although oleaster is widely accepted as an extremely stress-tolerant plant, information about its responses to chemical stress is scarce: available data are limited to soil components: nutrients, aluminum, salt, and acidity/alkalinity [16]. This work describes the first study of the physiological and biochemical responses of oleaster to elevated ammonium levels. A preliminary account of this investigation has been presented at the 12th Alps-Adria Scientific Workshop, Opatija, Croatia, in April 2013 [17].

Ammonium treatment *in vitro*

Freshly developed rootless shoots (approximately 45 mm shoot length) were transferred into WPM media supplemented with 20 g L^{-1} sucrose and $(NH_4)_2SO_4$ with a concentration series of 0, 20, 50 and 100 mM. Plantlets were incubated for 25 days using fluorescent lamps (3000 lux, 16/8 photoperiod). Digital images were taken and the pictures were subjected to image processing (ImageJ software, NIH, Bethesda, USA) [18]. Lengths of shoots and roots were measured during image analysis.

Ammonium treatments *in vivo*

Young leaves with petioles were cut from native tree and exposed to $(NH_4)_2SO_4$, NH_4Cl, and NH_4NO_3 by putting their petioles into aqueous solutions supplemented with NH_4^+ at a concentration series of 0, 25, 50, 200, 400 mM, and keeping them under daylight for 48 hours.

Enzyme extraction

Total proteins were extracted as follows: 0.5 g plant tissue were grounded with liquid nitrogen and homogenized in 3 ml homogenizing buffer (1M Tris-HCl – pH=7.8; 1mM Na_2EDTA; 7.5% polyvinylpyrrolidone). The suspension was centrifuged at 10000 g for 20 min at 4 °C and the supernatant was used for enzyme assays.

Glutathione S-transferase (GST) activity

GST activities were determined spectrophotometrically by measuring the formation of the reaction product conjugate molecule at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate [18]. The reaction mixture contained 0.1 M Na-phosphate buffer (pH 6.5) with 1 mM Na_2EDTA; 18.33 mM 1-chloro-2,4-dinitrobenzene; 19.8 mM reduced glutathione; and 50 µl plant extract in a volume of 2 ml. Assays were performed at 30 ºC. Enzyme activities were calculated as fresh weight activity (activity g-fresh weight^{-1}).

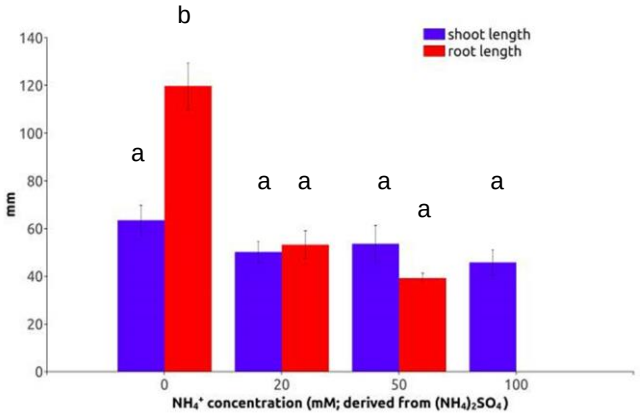

***Figure 2**. Effect of ammonium-sulfate (0 to 100 mM) on shoot and root development of oleaster (Elaeagnus angustifolia). Rootless plantlets (35 mm height) were put on WPM media supplemented with 2% saccharose and a concentration series of $(NH_4)_2SO_4$. After 25 days shoot and root lengths were measured using image processing software. (Bars represent the mean ± SEM of three replicates).*

Glutamate dehydrogenase (GDH) activity

GDH activity was determined by deaminating reactions according to Skopelitis *et al.* [20]. The standard deamination reaction mixture contained 100 mM Tris-HCl, pH 9.3; 100 mM L-Glu; 1 mM $NADP^+$; 0.5 mM $CaCl_2$; enzyme solution, and deionized water to a final volume of 2 ml. Assays were performed at 30ºC. Absorption change was measured at 340 nm using a Shimadzu-1301 UV/VIS spectrophotometer. Enzyme activities were calculated as fresh weight activity (activity g-fresh weight^{-1}).

Chlorophyll content determination

Chlorophyll A and B contents of oleaster leaves were determined with a Shimadzu-1301 UV/VIS spectrophotometer according to Porra [21].

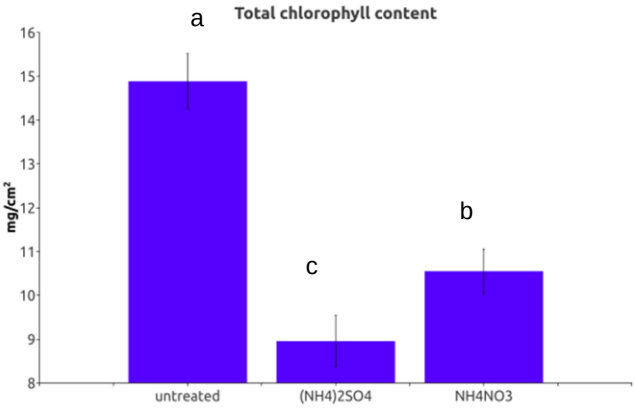

***Figure 3**. Total chlorophyll contents (ChlA + ChlB) of detached oleaster (Elaeagnus angustifolia) leaves. Shoots were treated with ammonium sulfate or ammonium nitrate (for 72 h) setting the final ammonium concentration to 400 mM. (Bars represent the mean ± SEM of 10 replicates)*

Statistics

At least three independent parallel experiments were carried out in each case. The significant differences between mean values were evaluated by Student's *t*-test. Differences were considered to be significant at P=0.05.

Results and discussion

Phytotoxic effects of ammonium on oleaster

In vitro test were performed to determine the lethal and sublethal concentrations of ammonium sulfate on oleaster *in vitro*. Root and shoot development from calli were inhibited by all concentrations of ammonium-sulfate investigated. Shoot growth was less affected: at 100 mM ammonium concentration no root development were observed (Figure 2). Chlorophyll A and B were also reduced significantly at higher ammonium concentrations (Table 1).

Phytotoxic effects of ammonium on detached leaves of oleaster

The direct effect of ammonium were monitored and characterized in this set of experiments by comparing the effects of ammonium sulfate with ammonium-nitrate.

***Table 1**. Chlorophyll content of detached young oleaster (Elaeagnus angustifolia) leaves treated with different salts of ammonium. Final concentration of NH_4^+ was set to 400 mM. Samples were taken 48 hours after treatments (n=4)*

	Chl A mg/cm^2 ± SEM	Chl B mg/cm^2 ± SEM
untreated	15.32 ± 0.98	4.43 ± 0.19
$(NH_4)_2SO_3$	11.76 ± 1.94	3.70 ± 0.44
NH_4Cl	12.59 ± 0.53*	4.14 ± 0.25
NH_4NO_3	11.85 ± 0.48*	4.07 ± 0.33

*Indicates statistical differences compared to untreated samples at p=0.05

Phenotypic appearance of the shoots was different only at 200 and 400 mM ammonium concentrations, where the leaves became dry and breakable. The decreases of chlorophyll content indicated the toxicity of 400 mM ammonium independently of the application as either nitrate or sulfate salts (Figure 3).

Induction of plant GST by ammonium has not been described previously. Although this enzyme may have a direct role in the maintenance of the redox balance of the cell (disturbed by ammonium) [22], a general stress-response seems to a simpler explanation. The 72 hour exposition time chosen was based on the results of preliminary experiments. The findings show that both ammonium-salts induce the GST activity even at low concentrations (Figure 4).

GDH activities were assayed by following the conversion of glutamate to 2-oxoglutarate. Although induction of GDH by ammonium has been observed previously [23], in our experiments ammonium-nitrate did not influence the enzymatic activity (only slight induction were observed at 200 mM NH_4NO_3), while ammonium-

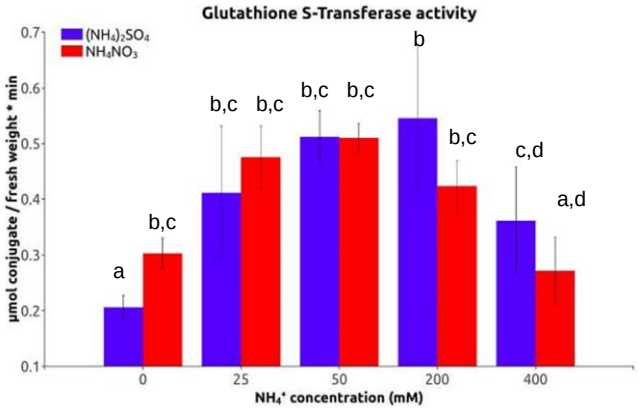

***Figure 4**. Glutathione S-transferase (GST) activities measured in detached oleaster (Elaeagnus angustifolia) leaves. Detached leaves were treated with a concentration series of ammonium-sulfate or ammonium-nitrate for 72 h. (Bars represent the mean ± SEM of three replicates)*

sulfate markedly decreased it (Figure 5).

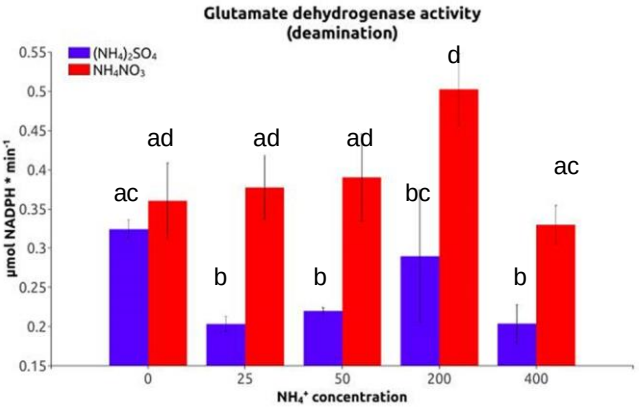

***Figure 5**. Glutamate dehydrogenase (GDH) activities measured in detached oleaster (Elaeagnus angustifolia) leaves. Shoots were treated with a concentration series of ammonium-sulfate or ammonium-nitrate. (Bars represent the mean ± SEM of three replicates)*

Conclusions

The effect of ammonium on oleaster was studied to reveal the basis of the extreme stress tolerance. Based on the results a breeding program should be initiated for producing stress tolerant trees with major economic importance (e.g. poplar or willow). These trees with extreme stress tolerance could be effectively used for green energy production and soil rehabilitation.

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