Effects of Mixtures Made of Alperujo (Solid by-product of Olive Oil Extraction) and Soil, on Bioindicators Development (*Eiseniaandrei* and *Medicago sativa)*

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Abstract

Solid wastes resulted from the olive oil extraction of represent an important environmental problem in Catamarca where they are generated in huge quantities, within short periods of time. These wastes are phytotoxic materials, but they contain valuable resources such as large proportions of organic matter that could improve soil productivity of these regions. The aim of this study was to determine the impact on soils, caused by the addition of alperujo to soils in different proportions, by using *Medicago sativa* and *Eisenia andrei* as bioindicators. Mixtures were typical soil of the Central Valley of Catamarca with the addition of fresh alperujo in different proportions (0-30%). The assays were performed under controlled conditions of temperature and humidity for 10 days. Adult worm survival and the presence and number of other stageswere analyzed. Additionally, *Medicago sativa* germination percentage and plant growth parameters were also recorded. Results revealed the survival of around 100% of the adult worms at 5 and 10% of alperujo/soil mixtures. Cocoons were found in soil without alperujo and alperujo/soil mixture 5 %. The germination of *M. sativa* reached the 40 and 10% in alperujo/soil mixtures of 20 and 30% respectively; whereas the 5 and 10% mixtures showed no inhibition of the germination. The emergence was affected from 10% alperujo/soil mixture.The plant height decreased with the concentration of alperujo, being the plants exposed to the 30% the most affected. We conclude that 5% of alperujo in these soils do not produce substantial changes on bioindicators, however, other studies would be needed to assess environmental waste.

INTRODUCTION

Olive cultivatedarea inArgentinais around115,000h whereas in the Catamarca province it is 30,000 h. Of total Catamarca area near to 63.3% corresponds to a non-producing area and 58% to producing one. Itenvisionsthat incoming years, this provincewill bethe largest Argentine olive producer (Atlas Catamarca, 2012).

Farming and milling olive processes have experienced in recent years an intense process of modernization that has contributed to increase fruit production and oil quality. Millers haveadoptedageneralizedcontinuous extractionsystemusing a two-stagehorizontal centrifugation. This systemhelps to eliminate a high percentage of the residual effluent (alpechin) that causes many problems to managementof mills. Thetwo-phasesystemgenerates an extracted raw material (pomace) with moisture of 50-70% (Intelligent Energy Europe, 2008). This waste material is called alperujo and contains a mix of alpechin and olive marc.

Alperujo production is a very important trait during olive oil extraction and becomes difficult to manipulate. The alperujo to olive ratio is 1:1 (González Fernández et al., 2003). The generation of alperujo has become in a serious environmental pollution problem, and then the finding of alternative new treatments is crucial to clean-up the environment. Available information regarding the direct soil disposal of the alperujo is scarce and controversial. Although the highorganicmatter contentofthisresiduemay be highlybeneficialfor agricultural practices, its residual oil content can increases oil hydrophobicity and reduce the water retention (Niaounakis and Halvadakis, 2004). A direct application of untreated alperujo to soils can produce either positive or negative fertilizer effects. Positive effectsare related tohigh mineral concentrations especially potassium, and also by its potential capacity to mobilizesoilions. For example, applicationof untreated alperujocan increase the wheat cropyield (Lopez-Pineiro et al., 2007). Negative effects of the alperujo have been associatedwiththe high C/N ratio, whichcauses disequilibrium of the soil nutrientbalance and so it must beappliedwith an additional nitrogen source. Moreover, alperujo also contains phytotoxic compounds such as polyphenols (Paredes el al., 1999, Thompson and Nogales, 1999).

Faced the problem of direct application of fresh untreated alperujo, it is necessary to define indicators for soil quality under these conditions. According to the Society ofAmericanSoil Science Committee the soil qualityis defined as: "the functional capacity of a specific kind ofsoilto supportplant or animalproductivity, maintain orimprove the quality ofwater and air, and sustain the healt hand human settlement, with limits set by natural ecosystem management" (Karlen et al., 1997). Soil qualityincludes both production capacityandenvironmental protection.Specific functions of soil qualityinclude: capture, hold and release of nutrientsandother chemicals. They also include grasp, hold and release water to plants and underground reservoirs, as well as maintain an adequate habitat for the soil biological activity (Brejeda and Mooman, 2001). Unlike water and air, soil has no defined standards due to its variability. Direct indicatorscommonlyused to soil characterization correspond tophysical,chemicaland biological traits. The relevanceofbiologicalindicators is giving by their ability to reflectchangesin the soil capacity andfunctions. Soil biological indicators generally refer to the abundance of organisms and products because they are more sensitive and valuable in the interpretation of the dynamics of organic matter, giving rapid response to changes in ground handling, and thus they are sensitive to environmental stresses and easy to measure (Bandinck and Dick, 1999, Dalurzo et al., 2002). According to the NationalSoilResource Conservation(NRCS, 2004), biological indicators, among otherqualities, should be easy to measure, reflect changes insoil functions, includingbiological propertiesand to beapplicableunder field conditions. Indirect indicators to soil characterization are referred toproductive capacityof the soil. To use of alperujo is needed to know the soil-alperujo mixing proportions to get an optimum composting process, proper time to field translation and when it is not toxic for soil flora and fauna.

Ecological niche of the earthworms characterizes them as very important soil organisms and can be used as environmental bioindicators. *Eisenia fetida* and *E.andrei* earth worms are used in manycountriesto performtoxicitytests required forpesticide registration. Thesespecies are known as Californian red worms and can breed easily. Tests performed with these earthworms are standardized and internationally accepted. Earthworm tests provide reliable information on relative toxicity of different chemical, allowing obtain a preliminary assessment of toxicity ranges and estimation of effects in response to a continuous exposure. Furthermore, it is interesting to mention that the development of certain plant species can be use either as bioindicator of the compost quality or as useful crops in the study area (OECD, 2006).Among these species it can be mentioned the alfalfa (*Medicago sativa*),which is a useful forage species. Likewise, legumes have been recognized as reliable bioindicators of hydrocarbon contaminated soils (RiveraCruzandGarciaTrujillo,2004). Therefore, we hypothesized that the adult survival number and cocoons presence of Californian red worms as well as the emergence and development of alfalfa, are soil sensitive and valuable biological indicators to interpret dynamics of the organic matter. The aim of this work was evaluate seedling emergency, biomass production, root/stem ratio and leaf number of *Medicago sativa* as well as adult survival and cocoons of *Eisenia andrei* occurring in a typical soil of the Central Valley of Catamarca province amended with different percentages of untreated fresh alperujo.

MATERIALS AND METHODS

Alperujo was collected directly from the plant oil extraction (EEA-INTA, Sumalao, Catamarca, 28°28’20”S, 65°43’59”W, 515 m a.s.l).Soil samples were taken from a horizon surface (30 cm depth) of a foothill that sustains a secondary succession of Arid Chaco Forest (San Fernando del Valle de Catamarca, 28°27'8" S, 65º45'1"W, 524 m a.s.l.). Soil type is sandy loam, free of rocks and loess with normal relief sluggish, moderate permeability and well drained. Soil-alperujo mixtures were made by adding 5%, 10%, 20% and 30% (v/v) of fresh untreated alperujo to a determined volume of soil. Analytical determinations were: texture by the pipette method, pH (aqueous extract, 1:2.5), electrical conductivity (EC), total organic carbon (OC) according to Walkley and Black (1934), organic matter (OM) according to Jackson (1964), total nitrogen by Kjeldahl method (Bremner and Mulvaney, 1982), available phosphorus according to Watanabe and Olsen (1965), calcium carbonate by Collins calcimeter, sodium and potassium by photometrically flame, calcium and magnesium by titration with EDTA and sodium absorption ratio (SAR).

Earthworms were placed in 10-cm PVC tubes (12-cm diameter) according to methodology described by Sainz et al. (2000) and maintained under controlled conditions during 10 days. To soil-alperujo mixing assays (250 g), were realized with foothill soil mixed with fresh alperujo to get a final concentration of 5%, 10%, 20% and 30% in plastic containers. Each container was added with 10 adult earthworms (with clitellum), with three replicates per treatment. A control with soil without alperujo was also carried out. After 10 days, adult survival and presence and number of cocoons were recorded. To plant assays, containers filled with foothill soil containing 0% (control), 5%, 10%, 20% and 30% fresh alperujo were sown with *Medicago sativa* cv monarca sp INTA seeds (20 seeds for each container, three replicates for each treatment). After 10 days germination percentage, emergence percentage (number of seedlings emerging from soil surface), stem length (S), root length (R), total length (T), R/S ratio, fresh and dry biomass, and leaf number were measured. After fresh weight determination plant material was dried at 60ºC to constant weight and weighted again to obtain the dry biomass. Prior to sown, a seed germination test was performed by using the Sobrero and Ronco (2004) procedure.

Statistical analysis: differences among treatment were analyzed by one-way ANOVA, taking $p<0.05$ as significant according to Tukey’s Multiple Range Test,and were also used Dunnett’s procedure. Pearson’s correlation was applied to analyse the dependence of soil chemical parameters to alperujo/soil mixtures concentrations during treatments, using the software Excel for Windows. All significance tests were done at a 95% confidence level (P = 0.05).Student's *t* test (P <0.05) were realized for soil analytical parameters to show significantly different respect to control (0%).

RESULTS AND DISCUSSION

Soil electrical conductivity (EC) increased with increasing alperujo concentration (Table 1). EC value was lower than 2 dS m^{-1} (non-saline) in soils containig 0%alperujo, while that soils containig5to20% alperujo were less than 4 dS m^{-1} (slightly saline). Moderately saline or saline (4.1 to 8.0 dS m^{-1}) was 30% alperujo/soil mixture.Statistical correlations, Pearson's Coefficient (r_p) were high positive correlation between EC value and alperujo increment in soil (r_p = 0.98, *P* = 0.05). Sodicity measured values indicated that experimental soil does not is a sodic type soil (SAR<13) (Sumner, 1995). Alperujo addition decreased the soil pH values of 8.36, 8.20, 8.10 and 7.30 (alkaline) for 0, 5, 10 and 20 % alperujo, respectively; wile pH value of 6.4 (weakly acid) was at 30% alperujo content (Table 1); exhibited high negative correlation (r_p = - 0.98, *P* = 0.05). Organic matter (OM) and organic carbon (OC) increased with the percentage of soil-alperujo mixture, exhibited high positive correlation, r_p= 0.95, P=0.05 for both parameters Relation of carbon content to total nitrogen content (C/N ratio) was increased with the presence alperujo in all concentration, showed a low correlation (r_p= 0.63, *P*=0.05). Soluble and exchangeable potassium increased with alperujo concentration, with a high positive correlation (r_p= 0.99, *P*=0.05).

Soil chemical parameters i.e. EC, pH, OM, OC and K, showed changes with increasing concentrations of alperujo. Compared to control, lowest changes were observed at 5% mixture concentration. But above 10% mixture concentration significant changes were observed for almost all analyzed parameters (Table 1). Significant positive and negative correlations were also observed.Diminished soil pH due lipid hydrolysis and increased of ECand organic load for spreading olive mill wastewater has been reported by Barbera et. al (2013).

Earthworms showed an adult survival of 100% up to 10% alperujo-containing soil, whereas non-survival adults over 20% alperujo concentration. Cocoons were only found in both 0 and 5% alperujo content (4 cocoons per 10 adults), while at higher alperujo contents there were no visible coccons (Figure 1).

Table 2 shows mean values of measured growth parameters of alfalfa seedlings developed in different soil-alperujo mixtures. The germination of *M. sativa* reached the 40 and 10% in alperujo/soil mixtures of 20 and 30% respectively; whereas the 5 and 10% mixtures showed no inhibition of the germination. Emergence percentage in alperujo/soil mixture of 5% not show change respect to control (soil without alperujo). The emergence percentage was decreased in soil-alperujo mixtures from 10%. Of interest, emergence percentage was decreased strongly at highest alperujo concentrations *i.e.*72, 87 and 90% at 10, 20 and 30% alperujo addition, respectively. Leaf number was also decreased in alperujo-containing soil. As can see in Figure 2 in soils added with 20 and 30% alperujo concentration cotyledon emergence was only found, while with 5 and 10% alperujo content a true leaf development was observed. Root and stem lengths were similar in control soil (R/S = 0.88, Table 2). In soils containing 5 and 10% fresh alperujo, root length be elongates and stem length decreasescompared with control. This fact enhances the root to shoot ratio (R/S) giving approximately 3-fold increase at 5 and 10% alperujo concentration. However, by overcoming this concentration of alperujo, a length decrease tendency of both root and stem was observed (Table 2). Correlation curves showed R^2 values higher than 0.70 for measured parameters (R^2= 0.75, 0.90 and 0.87 for root length, steam length and R/S) indicating that emergence and seedling development of alfalfa monarca INTA cultivar was sensitive to alperujo concentration in growth soil according to seed emergence and growth test of OECD states (OECD, 2006). Both fresh and dry biomass decreases as the alperujo percentage in the soil increases, giving with 10% of alperujo concentration nearly 50% reduction of both fresh and dry biomass when comparing with the control soil (Table 2). While that with 5% of alperujo the dry biomass decreased softly (16%). Correlation curves showed R^2 values higher than 0.70 for measured parameters (R^2=0.97 and 0.98).

Shoot is one the most affected organ by abiotic stresses, especiallyby saline one (Munns, 2008).Thus, decreased shoot length observed in this work could be related to EC increases.

Justino et al. (2012) shown that olive mill wastewater are highly toxic not only to microorganisms, but also to invertebrates with intermediary position on trophic chains (e.g., *D. magna*, *D. longispina*, *B. calyciflorus*) as well as to primary producers (plants and algae) affecting the sustainability of receiving systems. According to our results both *M. sativa* and *E. andrei* are scarcely affected by a 5% alperujo mixture concentration allowing that both cocoons and seedlings stay alive. But these latter showed a slight dry biomass decrease and some morphological alterations. In fact, both species could be used as bioindicators of alperujo phytotoxicity.

Since nutrient supply found in our experiment was substantially similar to described by Monetta et al. (2012) for a direct application of alperujo to the soil, we assume that toxic effects of polyphenols could cause the observed reductions of analyzed biomarkers (Della Greca et al., 2001, El Hajjouji et al., 2007).

CONCLUSION

In this closed system where no leach soil occurs, a 10% alperujo concentration seems to be a critical level. At this concentration,earthworms survivedbutcocoons were not found. Further, the emergence of seedlings and dry weight strongly decreased, while the chemical parameters of soil showed significant changes when comparing with control soil. From this concentration both *M. sativa* and *E. andrei* were severely affected. Hence, we conclude that 5% of alperujo in these soils do not produce substantial changes on bioindicators, however, other studies would be needed to assess environmental waste.

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Tables

Table1: Soilanalytical determinations

	Soil mixtures				
Alperujo in soil (%)	0	5	10	20	30
E C (dS/m)	1.99	2.19*	2.42*	3.34*	4.34*
pH	8.36	8.20*	8.10*	7.30*	6.40*
N (%)	0.17	0.15	0.17	0.20*	0.23*
Organic Matter (%)	2.68	2.93	4.12*	4.59*	5.06*
OrganicCarbon (%)	1.60	1.70	2.39*	2.66*	2.95*
C/N	9.48	11.33*	14.06*	13.30*	12.82*
K (meq/L)	5.12	5.40	8.90*	14.40*	18.22*
Na (meq/L)	5.10	5.20	6.20*	6.40*	6.40*
SAR	1.60	1.60	1.60	1.20*	1.10*

Values are mean of three independent experiments. *In the same line are significantly different respect to control (0%) (P<0.05) by the Student's *t* test.

Table 2: Mean value ofquantified variableson alfalfa plants developed in different amount ofalperujo in soil

	Alperujo in soil (%)				
	0	5	10	20	30
Root length (mm) (R)	70.00	113.75*	149.10*	41.20*	19.60*
Stem length mm (S)	79.15	47.90*	38.65*	22.00*	19.50*
Total length (mm)	149.15	152.65*	197.00*	63.20*	19.55*
Root/Stem	0.88	2.95*	3.12*	1.87*	1.01*
Freshbiomass (mg)	74.25	55.85*	37.20*	27.40*	17.32*
Drybiomass (mg)	6.80	5.70*	3.60*	2.60*	1.65*
N° leaves	2.05	1.15	1.00	0	0
Germination (%)	100	98	94	40*	10*
Emergence (%)	95	90	28*	13*	10*

Values are mean of three independent experiments (n=60). *Values in each file are significantly different respect to control (0%), at a levelof significance of 5% (Tukey and Dunnet tests).

Figures

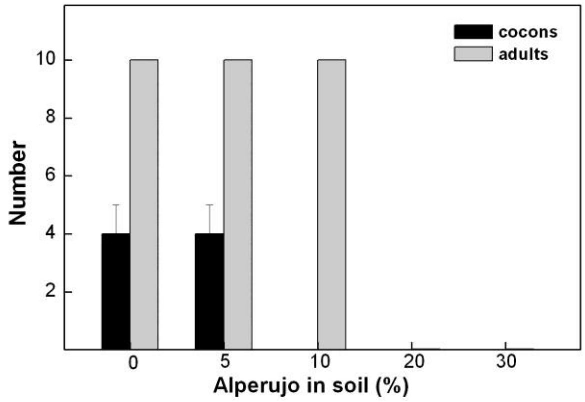

Figure1. Number ofadult earthworms andcocoons in alperujo/soil mixturesafter 10 days.Values are mean ± SD of threeindependentexperiments.

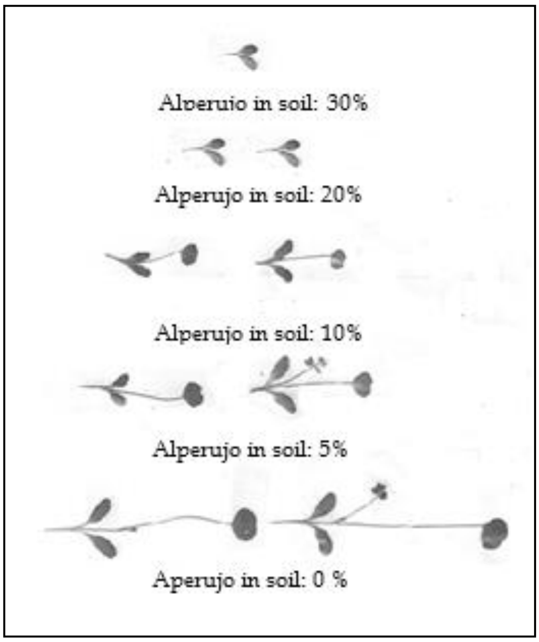

Figure2. Aerial portion of alfalfa seedling of 10 days ofdevelopment in mixturesof soil-alperujo