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SUMMARY

The biological activity of humic matter extracted from pasture and forested alpine soils, located in a unique climatic area, was investigated with *Picea abies* seedlings and *Zea mays* roots. The humic extracts were characterized by ^{13}C-NMR-spectra and by the amount of indole-3-acetic acid (IAA). Results evidenced that the forest humus, endowed with a higher phenolic C and IAA content, exhibits a better influence on peroxidase activity, esterase and peroxidase polymorphism and redox membrane activity.

KEYWORDS:

Humic matter, NMR spectra, IAA content, peroxidase and esterase activities, NADH oxidase.

INTRODUCTION

lt is well known that humic substances may affect plant metabolism, the transport of nutritive ions in plant roots and the mechanisms of plant growth regulation (1, 2). The nature of the response often depends on the concentration of the humic substances, and high concentrations are usually inhibitory. The range of the optional concentration and the precise effect varies between different plant species (1). These are also reports that the exact response depends on the source of the humic substances (2). The biological effect of the humic substances is influenced also by the molecular weight, and the major activity is related to the low molecular weight fraction.Ilt has been shown that the low molecular fraction is taken up by plants both actively and passively, whereas the high molecular weight (> 50,000 daltons) are taken up only passively (1).

It is therefore likely, that only the low molecular humic substances are taken up by a mechanism dependent on metabolism irrespective of whether they are derived from high and low molecular weight (1).

Recent studies (3) verified that humic matter contains indole-3-acetic acid (IAA), even if the concentration of IAA is not enough to justify the overall auxin-like activity.

Moreover, humic substances can accept electrons from a wide spectrum of donors and transfer these electrons to a wide spectrum of acceptors, so that humic substances may possibly interact with the surface redox activity of plasma membranes (4). It is known that the plasma membrane redox enzymes can transfer electrons from apoplastic or cytoplasmic donors to different types of acceptors. This transport has also been influenced by auxin, suggesting that the redox activity may play a role in hormone action (5, 6).

The aim of this paper is to evaluate the influence of humic substances extracted from pasture and forested soils on invertase, esterase and peroxidase enzymes and on redox activity in plants.

Invertases affect the mobilization of carbohydrates in plant seedlings and are involved in the plant growth (7), esterases are enzymes involved in embryogenesis (8), while peroxidases are ubiquitous enzymes in plants. Peroxidases are involved in numerous developmental programs and environmental stimuli like stress, pathogen attack, and fruit ripening. Peroxidases might also influence plant growth, as these isoenzymes may decarboxylate IAA in vitro (8).

MATERIALS AND METHODS

Study sites and soils.

Eight experimental sites, located on the plateau of Monte Bondone (Viote), central Alps (Trento, Italy), have been designated as Haplic Luvisols (9). They were chosen on the basis of having a homogeneous morphology and parent material.

Four soils are grassland *(scorzonero aristalae - agrostidetum tenuis)* and four, under reforestation *(Picea abies (L.) Karsten),* of about 50 years old. Samples were collected from the main rooting horizons (Ah) of each experimental soil and then air dried, stored and analysed by usual procedures (10).

Preparation of humic solutions

Humic substances were extracted with 0.1 N KOH (1:10, w/v) by shaking the suspension for 16 h at 50 °C. The mixture was centrifuged at 5,000 x g for 30 min, and the supernatants were dialyzed in Visking tubes (15,000 mol wt cut-off) against distilled water to pH 6.0. The solution was filtered through a column of Amberlite IR 120 H^+ form (2).

Nuclear magnetic resonance measurements

Quantitative ^{13}C-NMR spectra of the humic substances were obtained as described by Piccolo et al. (11).

Determination

Determination of IAA. Quantitative determination of IAA in humic substances was carried out using an enzyme immunoassay, Phitodek-IAA (Sigma Chemical Company, St. Louis, Missouri USA), according to producer instructions.

Invertase, peroxidase and esterase activities

The Norway spruce seedlings were grown in sterile conditions for 12 days in Petri dishes containing filter papers (Whatman n° 3) wetted with 0.1, 1, and 2 mg C L^{-1} of the extracted humic substances or with IAA or gibberellic acid (GA) or with 1 mM $CaSO_4$ (control). The invertase activity was evaluated according to Arnold (12), esterase activity measured as described by Junge and Klees (13) and peroxidase was determined according to Putter (14) in the 12 day-old seedlings. The esterase and peroxidase isoenzymes were resolved on polyacrylamide gel according to Laemmli (15). The effects of humic substances on surface redox activity were tested on 4 day-old seedlings of *Zea mays* (L.) *var. Mitos.* Maize seeds were germinated in the dark at 26 °C over an aerated 1 mM $CaSO_4$ solution. The redox activity was determined according to Pinton et al. (4). All biological data recorded were the means of three replicates, and the standard deviation was always within 5%.

RESULTS AND DISCUSSION

Nuclear magnetic resonance spectroscopy (NMR) is proving to be a powerful tool in the characterization of organic and humic matter. Data (Table 1) do not reveal marked differences between pastures and forested soils and this could be related to the young age of reforestation. Even if the differences among the carbons are not statistically significant, a trend may be evidenced. Aromatic, peptide and carbohydrate C reach higher values in pasture and this is related to the different composition of litters (16); while aliphatic C is higher in reforestation, probably because of the higher content of lignin derived from the needles (17). The phenolic and carboxyl C of reforested sites are higher than pasture as found also by Fyles and Fyles (18).

In a previous paper (19), comparing the NMR spectra of pasture and forest, we noticed a similar trend for aliphatic, carboxyl, peptide and carbohydrate C and a different trend for aromatic and phenolic C. This behavior could be related to different climatic conditions and vegetal associations which create a different ecosystem.

The effectiveness of the acidity of the humic substances is known to influence plant metabolism, moreover, the phenolic component is considered to have a hormone-like activity, which exerts a direct action on plant growth (20), as phenolics exert a protective role towards the auxin, retarding the IAA oxidation (21). This trend is confirmed by the IAA content in the humic substances (Table 1), that shows a higher level in the reforestation.

Comparing the data regarding the biological activity of humic substances it appears that both the humus from pasture and from reforestation sites significantly affects the activity of the in *vitro* peroxidase (Table 2). In particular, in the pasture the average stimulation is 185 % (control = 100 %) while in the reforestation is 138 %.

With regards the esterase and peroxidase electrophoretic patterns (figure not reported), our results show that humic substances and IAA enhanced the isoenzymatic polymorphism in Norway spruce seedlings with reference to the control, suggesting that humic substances exhibited an auxin-like activity. For invertase activity (Table 2) the results show that the reforestation is similar to the control, while the pasture is different both to the control and the reforestation.To determine the redox activity, roots of intact maize seedlings were used, as this plant is suitable as species to study the response of plants to humic substances (22).

Maize roots (Table 3) oxidize external NADH and reduce exogenous ferricyanide ($K_3Fe(CN)_6$) at a rate of 0.07 mM gfw^{-1} min^{-1} and of 0.58 mM gfw^{-1} min^{-1}, respectively (control). The rate of NADH oxidation was about 19-fold times higher when assayed in the presence of ferricyanide;

ferricyanide reduction increases from 0.58 mM gfw^{-1} min^{-1} to 2.05 mM gfw^{-1} min^{-1} with the addition of the electron donor. Humic substances differently affected oxidoreductase activities in maize roots.

All the humic substances decreased the rate of NADH oxidation and ferricyanide reduction when agents were present separately and NADH oxidation measured in the presence of ferricyanide. The trend was not clear when the reduction of ferricyanide was measured in the presence of NADH. The inhibition of NADH oxidation in the presence of humic substances from reforestation sites is higher than pasture. In the presence of ferricyanide the trend is confirmed but the level of inhibition is lower. All humic substances inhibited the reduction of ferricyanide in a similar way. The inhibition of NADH oxidase activity observed could lead to a delay in cell wall formation and might be regarded as part of the complex mechanism, by which humic substances enhance plant growth (4).

The IAA level, the stimulation of the *in vivo* peroxidase and esterase activity and the redox activity show the best biological effect of humic substances from the forested soils. The biological activity of humic matter may be related to the content of phenolic groups and to the IAA level. In particular phenolic groups, retarding IAA oxidation, play an important role in influencing plant growth.

TABLE 1
Organic and humic matter

	Relative intensity (total area %)					
Soils	**(0-48) Aliphatic C%**	**(48-105) Peptide/Carbohydrate C%**	**(105-145) Aromatic C%**	**(145-165) Phenolic C%**	**(165-190) Carboxyl C%**	**IAA %**
Pasture	12.92a	37.05a	23.5a	6.43b	20.08a	0.0081b
Forest	13.62a	36.18a	21.83a	7.63a	20.75a	0.0198a

Data with different letters differ significantly (p = 0.05).

TABLE 2
Biological activity of humic substances in humus from pasture and reforestation sites.

	Enzymatic activities					
Soils	**Invertase mg sucrose gfw^{-1} h^{-1}**	**%**	**Esterase OD $gfw^{-1}min^{-1}$**	**%**	**Peroxidas ΔOD gfw^{-1} min^{-1}**	**%**
Control	135.5b	100	0.45a	100	0.016c	100
Pasture	164.7a	113	0.38b	85	0.029a	185
Forest	144.2b	106	0.47a	105	0.022b	138

Data with different letters differ significantly (p = 0.05).

TABLE 3
Oxidation of external NADH and reduction of exogenous ferricyanide by maize roots.

	Rate of NADH oxidation (mM gfw^{-1} min^{-1})		Rate of $K_3Fe(CN)_6$ reduction (mM gfw^{-1} min^{-1})	
Soils	**in absence of $K_3Fe(CN)_6$**	**in presence of $K_3Fe(CN)_6$**	**in absence of NADH**	**in presence of NADH**
Control	0.07c	1.35a	0.58a	2.05d
	0.06d	1.28a	0.42b	2.27c
Pasture	0.07c	1.03b	0.32c	1.96d
	0. 15a	1. 10b	0.3 Ic	2.56b
	0.003f	1.07b	0.32c	2.84a
Forest	0.02e	1.03b	0.45b	1.78e
	0. 13b	1.00b	0.34c	1.74e

Data with different letters differ significantly (p = 0.05).

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