Effects of two humic acids on growth of winter oilseed rape and wheat

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Abstract: Developing new fertilisers respectful of the environment is a key point to manage the cultural practises changing. Among all the biological molecules that could act as fertiliser, we choose to focus on two humic acids (HA), one extracted from peat (HA-P) and the other from Leonardite (HA-L). Firstly, physico-chemical properties of the two HA have been determined using ^{13}C NMR, HPSEC and potentiometric titration. These analyses revealed that the two HA present structural differences especially concerning molecular weight and aliphatic characters. Secondly, in order to verify if these differences could induce contrasted biological activity, growth parameters have been monitored in hydroponic plants (wheat and rapeseed) incubated 4 weeks in Hoagland supplemented with HA-L or HA-P.
The results of the rapeseed cultures show contrasted effects for the two HA. Only HA-P treatment results in higher dry weight, chlorophyll content and Nitrogen Use Efficiency (NUE) compared to control. The contrasted biological activity of these HA-L and HA-P may be related to their different physico-chemical properties but also depend on the treated plant.

INTRODUCTION

Changing cultural practices is essential to match regulatory and social pressures for the development of agriculture more respectful of the environment. Improving breeding and varietal selection are the main tracks to obtain high yielding varieties in response to low levels of fertilisation (especially nitrogen). However, other strategies to stimulate the uptake of mineral elements in soil by plants are booming. Among these strategies, research is directed toward biological molecules such as humic acids. These main components of soil organic matter are already known to improve dry weight, chlorophyll content and yield of treated plants compare to control plants [3, 6, 9, 10, 11]. In contrast, there is no evidence of studies comparing the effect of humic acids supply in different species.
Thus, in our study, we compare the effects of two humic acids on the growth parameters of two different plants: a monocotyledon (wheat) and a dicotyledon (rapeseed). The work described here is a part of a larger project named "AZOSTIMER" whose aim is to increase nutritional efficiency in crop plants using new biological substances (such as humic acids) in order to limit the current using of chemical fertilisers.

MATERIAL AND METHODS

Characterisation of physico-chemical properties of humic acids

Humic acids origins: The two humic acids (HA) are extracted and isolated following the IHSS procedure [13, 14]. HA-P comes from peat and HA-L comes from Leonardite.
-^{13}C RMN ^{13}C RMN spectra were obtained on a Bruker Avance AV-400WB (9.4 T) spectrometer at 100.47 MHz using the cross-polarization magic angle spinning (CPMAS), with a spinning speed of 12 kHz, 90° pulse width, 30 ms acquisition and 4.0 s delay.
Hydrophobic and hydrophilic carbon were measured according to Canellas et al. [4], and aromatic degree ([112-160]/[0-112]) according to Canellas et al. [5].
- Determination of molecular size (MS): MS distribution was evaluated by high performance size exclusion chromatography. The chromatographic system consisted on a Waters 600 Controller pump followed by two detectors in series: a Waters 996 Photodiode Array Detector set at 400 nm and a Waters 2424 Refractive Index Detector (RI). Size exclusion separation occurred through a PL Aquagel-OH 30 column (Polymer Laboratories), preceded by a guard column with the same stationary phase. The overall molecular range of separation for this column is 100-300,000 Da.
For each sample, solutions of 800 ppm of carbon were prepared in 0.05 M $NaNO_3$. The injection volume of all samples was 100 µL. The eluent used was 0.05 M $NaN0_3$ (pH 7) and the flow rate was 1 mL/min. Void volume (V_0 = 6.65 mL) and permeation volume (V_p = 11.82 mL) were determined with polyethylene oxide of molecular weight (MW) of 43.250 Da and methanol respectively. To evaluate an approximate MW distribution from HPSE Chromatograms, a universal calibration was carried out. Curves of log J versus elution volume were obtained using polyethylene glycol and polyethylene oxide as standards of known MW. The parameter J id defined as the product of the intrinsic viscosity [η] and the molecular weight ($J_i = [\eta]_I M_i$). The Mark-Houwin-Sakurada equation relates [η] to MW as follows:

$[\eta] = K M^{a}$,were K and a are constants proper of each macromolecule, solvent and temperature. In this study we have used the value of K and a reported by Visser [16] for a soil humic acid ($K = 2.724x10^{-2}$ $mL.g^{-1}$ and a = 0.45).

Plants cultures, treatment and determination of physiological parameters:
Rapeseed: Seeds (Brassica napus cv. Capitol) were surface sterilised by soaking in alcohol 70° for 5 min and bleach (5% Chlorine) for 10 min. Seedlings were transplanted on tanks containing 20L of Hoagland solution with 1 mM NO_3^- (18 plants on each tank) during 4 weeks at 20°C with D/N cycle of 16/8h (Lamp Hortilux Schreder, 400 W, HS.TP4.23) in a greenhouse.
Wheat: Seeds (Triticum aestivum cv. Bermude) were germinated 10 days in dark on perlite. Seedlings were transplanted on tanks containing 15L of Hoagland solutions with 1 mM NO_3^- during 4 weeks at 25/16°C, 16/8h day/night in a greenhouse.
Humic acid (HA) input: HA were added to the nutrient solution at concentrations of 10 (data not shown) or 100 mg of organic carbon per L (mgC/L). Control plants were cultivated without HA input.
Dry weight (DW) determination: During the harvest, shoot and root of plants were separated, weighted (fresh weight, FW) and dried in oven dry during 48h at 60°C for DW determination.
An aliquot of DW was then crushed for N total analyses by IRMS
Nitrogen Use Efficiency (NUE): In this work, NUE is defined by the quantity of DW produced with 1g of nitrogen. It is calculated as follows: NUE = DW / QNtot
Leaf Chlorophyll contents: non-destructive measures of chlorophyll contents are performed using contact-free optical sensor Multiplex, manufactured by FORCE-A (Orsay, France).

Statistical analysis
Data presented were means of 10 replicates and analysed using the Student test with p = 0.05.

RESULTS AND DISCUSSION

Physico-chemical characterisation of humic acids
^{13}C RMN indicate that HA-P contains more O-alkyl groups, more aliphatic carbon and more carbonylic carbon than HA-L. These results suggest the presence of more O-alkyl-aliphatic unsaturated chains including C=O bounds in HA-P than HA-L. Moreover, HA-L presented more aromatic arrangements than HA-P. These results were reflected the hydrophobic index and aromatic degree that was higher for HA-L than HA-P. Apparent molecular weight (MW) of the two HA has been performed by HPSEC (Table I). The results indicate that HA-P as an apparent size distribution lower in average than HA-L. HA-P is composed of lower MW molecules than HA-L. Moreover, the potentiometric titration studies indicate that HA-P have total acidity higher than HA-L. This acidity is principally due to the high concentration of very weak acidic groups, which may include O-alkyl and O-aryl groups (data not shown).

Table I: Apparent average molecular weight and distribution for HA-P and HA-L.

	Main peaks (Da)	Interval MW (Da)	Area (%)
HA-P	$4.96 \cdot 10^4$	$2.05 \cdot 10^4 – 1.46 \cdot 10^5$	9
	$1.36 \cdot 10^4$	$9.46 \cdot 10^3 – 1.98 \cdot 10^4$	68
	$5.42 \cdot 10^3$	$1.50 \cdot 10^3 – 1.78 \cdot 10^4$	36
HA-L	$2.33 \cdot 10^4$	$9.53 \cdot 10^3 – 6.74 \cdot 10^4$	44
	$5.96 \cdot 10^3$	$5.97 \cdot 10^2 – 4.91 \cdot 10^4$	65

Thus, as described above, the two HA used in this study present very different physico-chemical properties: HA-L is a high molecular weight HA with aromatic cycles that result in a hydrophobic molecule. In contrary, HA-P is a low molecular weight HA with acidic aliphatic unsaturated chains.
These differences in physico-chemical structure can suggest that these two HA could present different biological activities. To clarify this aspect, the effects of the two HA on growth parameters have been studied in two different crop plants: rapeseed and wheat.

Effect of humic acids on physiological parameters of rapeseed and wheat
In order to study the biological activity of two HA (HA-P or HA-L), growth parameters of rapeseed (dry weight (DW), chlorophyll contents and nitrogen use efficiency (NUE)) have been measured after 4 weeks of treatment. Main results were presented in table II.
In response to HA-P treatment, all DW parameters increase. Indeed, compared to control plants, the total DW of rapeseed treated with HA-P increase by 34% and by 44% and 46 % for shoot and root

respectively. In addition, NUE is although enhanced, suggesting that rapeseeds can made more DW with the same quantity of nitrogen absorbed in response to HA-P. Finally, the HA-P treatment induced also an increase of chlorophyll content (+18%). These results suggest that treatment of rapeseed with HA-P enhance the growth in contrast to treatment with HA-L, which has no significant effect on all growth parameters (table II).

Table II: Physiological parameters (dry weight, chlorophyll content ant NUE) were measured after treatement with two humic acids (HA-P or HA-L) at 100 mgC/L. Values are in percentage of the control. Values in bold are significantly different from the control (Student test, p<0.05).

		HA-P	HA-L
Dry weight	total	**+34**	+7
	shoot	**+44**	+9
	root	**+46**	+3
Chlorophyll content		**+18**	-13
NUE		**+55**	+6

Whatever the plants treated (rapeseed or wheat), HA-L has no effect on the growth parameters monitored in this study. For rapeseed, these results are in line with previous studies which demonstrate that oil-producing plants are not affected by application of HA [15]. In contrast, these results are most surprising for wheat since previous observation reveal that cereals shows a moderate reaction in response to HA treatment [15]. Nevertheless, we have shown that HA-P present contrasted effects on the two plants. Indeed, HA-P increases all growth parameters monitored for rapeseed but it shows no effect on wheat.

Physico-chemical properties of HA and effects on rapeseed growth

Rapeseed shows a highly positive response to the application of HA-P. The enhancement of dry weight (especially root dry weight) and chlorophyll contents observed in response to HA-P treatment agrees with previous works showing several positive effects of HA application on growth of some plants such as corn [3, 4, 6, 10, 11, 12]. If we consider the chemical characterisation of HA-P and HA-L, these observations on physiological parameters suggest that lower apparent MW distribution and more aliphatic chains could support a better biological activity on the plant growth [1, 2, 4, 5, 8, 12].

CONCLUSIONS

HA effects depend on several parameters such as target plant (rapeseed versus wheat), physico-chemical properties of the HA (HA-P versus HA-L) but also HA dose used.

To use HA to complement or substitute chemical fertiliser currently used, following studies will aim at: (i) for wheat, verify if a higher concentration of HA could induce a significant effect on growth parameters, (ii) for rapeseed, the effects observed in hydroponic cultures will have to be confirmed in soil culture. Moreover, using a Microarray approach, we will investigate major the metabolic targets of HA-P in Brassica napus.

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