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A triglyceride from *Lupinus albus* L. seed with bio-stimulatory properties

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***In vitro* and *in vivo* bio-stimulatory activity of a *Lupinus albus* cv. Bethuana white Seed Suspension (SS) was previously documented using vegetable crops. This was repeated for two grain crops, maize and wheat, in an attempt to provide a rationale for the isolation and identification of the active compound(s) involved. In all cases, besides negative controls, the response to treatment with *SS* was compared to that of a commercial bio-stimulant, ComCat®. At an optimum concentration of 5 mg ℓ^{-1} SS significantly enhanced seedling growth and yield in both crops. Subsequently, activity directed liquid-liquid extraction of ground *L. albus* seed resulted in an ethyl acetate extract containing most of the bio-stimulatory activity. Two *in vitro* bio-assay procedures were employed to appraise the *in vitro* bio-stimulatory properties of semi-purified fractions as well as pure compounds contained in it. The highly active ethyl acetate extract was fractionated further by means of adsorption column chromatography resulting in eighty five fractions that were combined into twelve on the basis of similarities in thin layer chromatography profiles. Four of these fractions showed significant bio-stimulatory activity and were pooled. From this one compound was isolated, purified and identified as glyceryl trilinoleate by means of nuclear magnetic resonance spectroscopy.**

Key words:*Lupinus albus*, seed suspension, field trials, yield, NMR spectroscopy, glyceryl trilinoleate.

INTRODUCTION

In light of a sharp increase in world population predicted for the future, and the resulting uncertainty towards food security, a need exists to increase crop yields on available arable land (Heidhues, 2001). Further, due to consumer resistance towards the extended use of synthetic chemicals in an attempt to reach this goal, a rationale for natural product research, especially environmentally friendly natural bio-stimulants that have the potential to improve crop yields, exists. This is not a new concept as plant extracts containing growth promoting substances have been of interest to the agricultural research community since the early seventies in terms of the role they could play in addressing future food security issues (Blum et al., 1992; Suzuki et al., 2002). Several plant species with growth promoting properties have been identified in the past. For example, Channal et al. (2002) reported on the seed germination as well as seedling growth enhancement of sunflower and soybean by leaf extracts from three (*Tectona grandis*, *Tamarindus indica* and *Samanea saman*) out of the seven tree species investigated. Similar effects were reported by Terefa (2002) for *Parthenium hysterophorus* extracts on tef (*Eragrostis tef*) and by Neelam et al. (2002) for *Leucaena leucocephala* extracts on wheat (*Triticum aestivum*). However, none of these studies revealed that treatment with the different plant extracts had any effect on the final yield of the crops

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under investigation. The ideal break-through would be to identify a plant or plants that contain bio-stimulatory substances promoting not only seedling establishment but also yield and quality in agricultural crops. In search of the ideal candidate, we screened a large number of wild and domesticated South African plants for bio-stimulatory properties. Above average growth promoting activity was recognized in a white lupine, *Lupinus albus* L. cv. Bethuana White, Seed Suspension (SS). The ability of SS to promote seedling development in terms of coleoptile and root growth was previously confirmed in a number of vegetables under laboratory conditions (van der Watt and Pretorius, 2011).

In a previous study, van der Watt and Pretorius (2011) reported on the growth and yield response of selected vegetables to foliar treatment with SS under laboratory (*in vtro*) and field (*in vivo*) conditions. The main objectives of the underlying study were to establish: 1) whether seedlings of grain crops (wheat and maize) also respond *in vitro* to foliar treatment with SS in terms of growth, 2) and *in vivo* in terms of yield over one growing season in an attempt to provide a rationale for, 3) the isolation and identification of the active bio-stimulatory compound(s) contained in *L. albus* cv Bethuana White seeds. In the case of field trials, a recently commercialised bio-stimulant, ComCat®, was chosen randomly from other commercial products and used as a positive control at the optimum concentration recommended by the manufacturers (AgraForUm, 2010). ComCat® contains brassinosteroids as active ingredient (a.i.) and is registered as a plant-strengthening agent by the European Union. Claims made by the manufacturers on their website that were authenticated by literature reports on the effect of externally supplied brassinosteroids on plants, include vegetative growth enhancement (Lim and Han, 1988), especially root growth (Müssig et al., 2002) of selected agricultural crops as well as yield enhancement (Kamuro and Takatsuto, 1999; Khripach et al., 2000; Zullo and Adam, 2002). The latter is explained on grounds of improved utilization of soil nutrients by treated plants and improved flower bud formation (Schnabl et al., 2001).

Other advantages of ComCat® include improved crop quality (Prusakova et al., 1999) and the induction of resistance towards fungal pathogen infection in potato (Schnabl et al., 2001).

MATERIALS AND METHODS

Plant material

L. albus cv. Bethuana White as well as maize (*Zea mays* cv. PAN6043) and wheat (*T. vulgare* cv. PAN3377) seed used in laboratory and field trials during the 2009/2010 growing season were purchased from SENWES (South Africa). Cress (*Lepidium sativum*) seed used in laboratory bio-assays were kindly supplied by AgraForUm AG, Germany.

Other materials

Methanol, hexane and ethyl acetate was purchased from Merck (Germany) and sodium bicarbonate from Sigma (Germany). All organic solvents were of the purest grade possible. ComCat® was a grant from AgraForUm AG.

Preparation of a seed suspension (SS)

Five hundred grams of whole *L. albus* cv, Bethuana White seeds were initially broken into small fragments using a Retsch SM 2000 (USA) cutting mill and ground to a fine powder (particle size <100 µm) using a Freutsch (Germany) seed grinder. Subsequently, the powdered seed was drenched with 100% (v/v) ethanol that was allowed to evaporate at room temperature. This was repeated twice. Subsequently, the ethanol treated seed material was suspended in distilled water at 5 mg/L by agitating vigorously on a mechanical shaker.

Field trial layout

Maize and wheat trials were conducted in South Africa on a farm 20 km north of Kenilworth in the Bloemfontein district (29° 01' 00" S, 26° 08' 50" E) during the 2009/2010 growing season. In both cases, trials were laid out in a complete randomized block design. Plot sizes differed for the different crops according to the prescribed in row and between row spacing suggestions of the Agricultural Research Council (ARC, South Africa, Technical datasheet 2004). Fertilizer was applied based on soil analysis and excepted yield outcome according to the recommendation of the Agricultural Research Council (ARC, South Africa Technical datasheet 2004) for South African soils. Summarized details for each crop (Table 1), soil analysis (Table 2) and weather data (Table 3) are supplied.

Treatment of crops under field conditions

Test crops were separately treated with the seed suspension (SS) and a commercially available bio-stimulant, ComCat® (positive control), by means of foliar applications at the 3 to 5 leaf growth stage (V16 for both maize and wheat) (Meier, 1997) while standard fertilized plots (Table 2) served as a negative control. SS was applied at 5 mg ℓ^{-1} (7 g a.i. L^{-1}/ha^{-1}) (previously found to be optimum under laboratory conditions) (van der Watt and Pretorius, 2011) for both test crops and ComCat® at 100 g ha^{-1} for maize and 200 g ha^{-1} for wheat according to the specifications of the distributors in South Africa (Agraforum SA, 2010). For both maize and wheat, the spray volume was 1400 ℓ ha^{-1}. In all cases, a wetting agent, Solitaire (Villa Crop Protection South Africa 2009) was added at a ratio of 1:1000 (v/v). All plots received standard fertilizer at planting (Table 2) while standard disease and insect control measures were applied for each crop. Trials were conducted under rain fed conditions and replicated 8 times using a randomized block design.

Quantification of crop yields

After completion of the drying cycle, 20 ears per replicate were collected at random from wheat plants that did not form part of the harvested area, and the dry mass determined. Subsequently, dry kernels were removed from the ears, counted and weighed. Total grain yield per replicate was determined from a 15 m^2 (1.5 × 10 m) area harvested by means of an experimental plot harvester (Wintersteiger, Austria). The harvested area was from five rows in the middle of each plot in order to avoid any possible side effects.

Table 1. Field trial specifics and fertilizer requirements for maize and wheat, cultivated under rain fed conditions, based on expected yield outcome and soil analysis as recommended by the Agricultural Research Council, Bethlehem, South Africa (ARC, Technical datasheet 2004).

Row crop	Maize	Wheat
Cultivar	PAN 6043	PAN 3377
Replications	8	8
Between row spacing	1.5 m	0.45 m
Plot size	34 m^2	30 m^2
Plants/ha	± 17 600	± 55 000
Harvested area per replicate	12 m^2	15 m^2
Yield potential	4.0 ton/ha	2.5 ton/ha
Fertilizer/ha	**kg/ha**	
N	70	45-60
P	15-20	15
K	0	7-8

Table 2. Soil analysis conducted by the Agricultural Research Council, Bethlehem, South Africa (2010).

N (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Na (mg/kg)	pH (KCl)	Soil properties (%)			CEC ($cmol_c kg^{-1}$)
							Sand	Clay	Loam	
294.0	7.3	75.5	413	192.9	6.2	5.5	84	12	4	3.9

CEC = Cation exchange capacity.

Table 3. Minimum, maximum and average daily temperatures as well as average monthly precipitation and relative humidity during the 2009/2010 growing season in the region where field trials were conducted as supplied by the South African Weather Service (Pretoria).

Year	Month	Temperature (°C)		Precipitation (mm)		Relative humidity	
		Minimum (Tn)	Maximum (Tx)	Average	Total	Minimum (RHn)	Maximum (RHx)
2009	May	6.0	20.4	0.9	29.2	29.5	82.7
	June	4.9	16.7	0.7	19.6	37.4	84.3
	July	1.7	16.1	0.3	10	23.8	71.2
	August	4.8	21.0	0.3	8.7	20.8	72.1
	September	6.9	25.9	0	0	103.1	55.8
	October	12.2	26.5	3.4	97.4	25.5	79.4
	November	13.6	28.7	0.7	8.5	21.6	76.1
	December	15.3	32.8	1.9	57.6	14.7	77.5
2010	January	16.8	28.4	4.3	133.3	39.1	86.9
	February	16.8	29.9	1.5	34.9	30.1	83.1
	March	16.6	29.6	4	111.9	31.3	83.6
	April	10.9	23.9	1.3	39.8	36.5	86.5
	May	6.7	21.6	0.6	19.8	30.2	81.0

Final yield was expressed in ton/ha. Maize cobs from twenty plants per row and from the three middle rows (60 plants per replicate) were harvested by hand after completion of the drying cycle and when kernels contained 12% moisture. Subsequently, cobs were mechanically dehusked while care was taken that none of the kernels went astray. The dry kernel weight for each replicate was determined separately and expressed as ton/ha on the basis of an average plant stand of 17 600 per ha.

Activity directed bio assays

Two different bio-assays were employed to determine and quantify the bio-stimulatory activity of the seed suspension, crude extracts and active compounds. These included the effect on the respiration

rate of monoculture yeast (*Saccharomyces cerivisiae*) and its effect on seed germination and subsequent seedling development in terms of root length growth as well as either coleoptile or hypocotyl growth, depending on the test plant. Either one or both the bio-assays were used during activity directed extraction of active compounds from the *L. albus* seed suspension.

Assay 1: Activity directed bioassay on the respiration rate of monoculture yeast cells

A specially constructed glass respirometer with a short bulged section (reservoir) to contain the yeast cells and a long calibrated tube, closed at the top end to collect CO_2 gas was used in determining the effect of a *L. albus* seed suspension (van der Watt and Pretorius, 2011), crude extracts and active compounds on the respiration rate of monoculture yeast cells. Dry baker's yeast (0.8 g) was placed in the reservoir of the respirometer. Subsequently, 70 ml of each of the test solution containing 5 g ℓ^{-1} glucose to serve as respiratory substrate for the yeast cells was added to the respiremeter. The apparatus was tilted sideways to release air bubbles trapped in the dry baker's yeast and placed in a water bath pre-heated to 29°C. ComCat® was used as a positive control at the prescribed concentrations and distilled water served as a negative control. Carbon dioxide released by the yeast cells was measured in ml at 20 min intervals over an 80 min incubation period by reading the gas volume directly from the calibrated tube. Tests were replicated six times and laid out as randomised designs.

Assay 2: Activity directed bioassay on seed germination and subsequent seedling growth

Two sheets of germination paper (30 × 30 cm; purchased from Agricol, South Africa) were used to determine the effect of different test solutions on seed germination and subsequent seedling growth of different crops. A line, 10 cm from the top, was drawn on one sheet and 20 seeds spaced evenly on the line. A second sheet of germination paper was placed on top of the first and moistened with a specific test solution, distilled water (negative control) or different prescribed concentrations of ComCat™ (positive control). Both sheets of paper were rolled up longitudinally and placed upright in an Erlenmeyer flask containing distilled water, the test solutions or the ComCat® solution and kept at 25°C in a growing chamber in the dark. Seed germination, coleoptile or hypocotyl and root lengths were determined at 24 h intervals over a 96 h incubation period and replicated six times using a randomised design.

Isolation, purification and identification of the active compounds in SS

Activity directed liquid-liquid extraction

A modified method of Gamoh et al. (1989) was followed. Five hundred grams of ground *L. albus* seeds were extracted with 2 L of 100% methanol (1:4 ratio; m/v). This was repeated 10 times over a 5 day period. Every 24 h, the methanol crude extract was decanted, pooled and replaced with fresh methanol (v/m) (1). Subsequently, the same plant material was extracted with an ethyl acetate: methanol mixture (1:1; v/v) in the same 1:4 ratio (m/v) for another five days by following the same procedure as explained for methanol (2). The methanol and ethyl acetate: methanol extractants were combined (3), mixed and filtered under vacuum through a double layer of Whatman No. 1 filter paper using a Buchner funnel fitted to an Erlenmeyer flask connected to a suction pump. The combined filtrate was vacuum distilled at 35°C using a Buchi Rotavapor (Bibby Sterlin LTD, England) equipped with a cooled Liebig condenser in order to remove the bulk organic solvent. The combined concentrated extract was fractionated between ethyl acetate and water (2:1; v/v) by means of liquid-liquid extraction and the procedure repeated three times (4). Both the ethyl acetate (5) and water fractions (6) were concentrated by means of vacuum distillation and the water fraction further fractionated twice between hexane and 90% methanol (1:1; v/v). The hexane fraction (7) was subsequently concentrated by means of vacuum distillation at 35°C and the aqueous water extract fractionated twice between ethyl acetate (8) and a saturated sodium bicarbonate solution (1:1; v/v) (9). All nine of the collected SS fractions were tested for bio-stimulatory activities by means of the two described activity directed bio-assays.

In both bio-assays, all fractions tested were replicated six times by using randomised designs. Only the most active fractions were further purified using different chromatographic techniques in order to isolate, identify and elucidate the chemical structure of the active compound(s).

Qualitative thin layer chromatography (Q-TLC) of liquid-liquid extraction fractions

Using Q-TLC, all the liquid-liquid extraction fractions obtained as outlined earlier were spotted on a silica gel 60 F_{254}-aluminum backed and pre-coated plate (Mikes and Chalmers, 1979) in order to obtain a Q-TLC-profile of each fraction. 10 to 15 µg of each sample was loaded onto the plates at the baseline and developed in a saturated chamber using chloroform: methanol (95:5; v/v) (Gamoh et al., 1989) and 1 ml of glacial acetic acid as solvent system. Glacial acetic acid was added to the mobile phase to prevent the fractions from streaking on the plate. After drying the plates in a stream of air, using a common hair dryer, compounds were first detected under UV-light at 254 and 365 nm, stained with 10% (v/v) ethanolic sulphuric acid and placed in an oven at 100°C for 20 min (Wagner and Bladt, 1996). Only the most active liquid-liquid extraction fraction (ethyl acetate fraction 1, Figure 1) was concentrated to dryness *in vacuo* and subjected to column chromatography for further purification of the active compounds.

Activity directed adsorption column chromatography of ethyl acetate fraction 1

From the activity directed liquid-liquid extraction procedure (Figure 1), ethyl acetate fraction 1 showed the highest bio-stimulatory activity *in vitro* and was further purified by means of adsorption column chromatography using Silica gel 60 (Merck, Germany) as stationary phase. One hundred gram silica gel 60 (70 to 230 mesh) was mixed with 300 cm^3 of the mobile phase (chloroform: methanol; 98:2; v/v) to form a slurry that was poured into a glass column (200 × 10 mm) and allowed to settle for an hour under gravitation. 5 ml of the active ethyl acetate fraction, at a concentration of 516 mg ml^{-1} was loaded onto the column and eluded stepwise with 50 ml of a gradient solvent system of 100% chloroform followed by 50 ml each of different chloroform: methanol mixtures (v/v; 98:2, 95:5, 90:10, 85:15 and 80:20) at a flow rate of 1 ml min^{-1}. A total of 85, 5 ml fractions were collected. To ensure that all active substances were removed from the column, it was finally cleaned with two bed volumes of 100% chloroform followed by two bed volumes of 100% acetone and collected separately in bulk. After vacuum distillation, 10 µg of every third column fraction as well as the bulk chloroform and acetone fractions obtained after cleaning the column was spotted on a pre-coated silica gel TLC-plate and the compounds contained separated, as outlined earlier, using chloroform: methanol (10:1; v/v) as mobile phase. After developing the TLC-plates with 10% (v/v) ethanolic sulphuric acid at 100°C, those column chromatography fractions with similar Q-TLC profiles were combined.

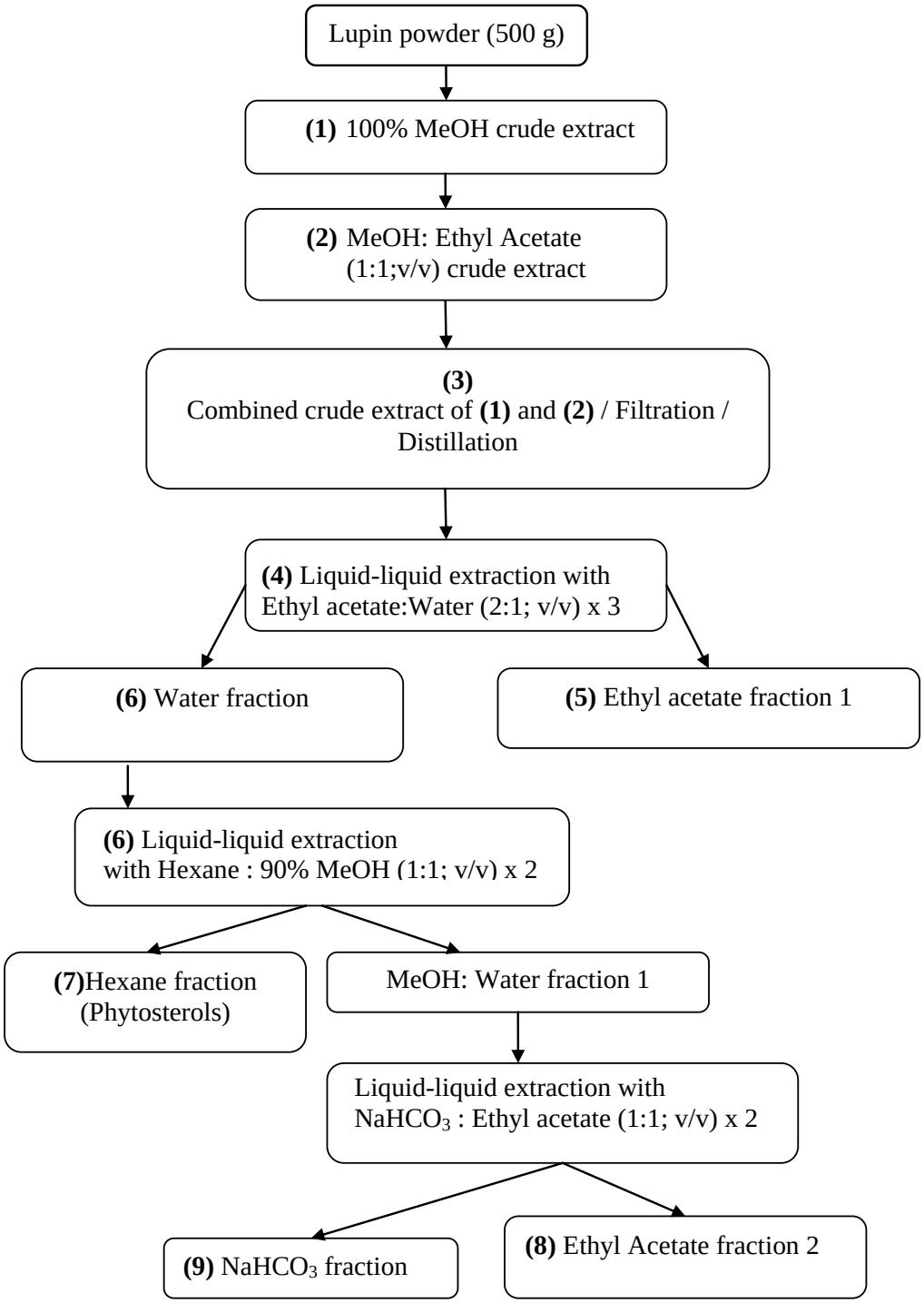

Figure 1. An outline of the procedure followed to extract and fractionate active compounds from *Lupinus albus* L. seeds (Gamoh et al., 1989).

In this way, twelve combined fractions were obtained that were subsequently concentrated *in vacuo* and subjected to the two bio-assay procedures in order to determine which fractions contained the bio-active compound(s). In both bio-assays, all fractions tested were replicated six times by using randomised designs.

Purification of active compounds using preparative thin layer chromatography (P-TLC)

Four of the twelve combined column chromatography fractions showing the highest bio-stimulatory activity were pooled and puri-

Table 4. Yield response of selected row crops to foliar treatment with a *L. albus* cv Bethuana White seed suspension (SS) under rain fed conditions. ComCat® was used as positive control. Untreated plants receiving only standard fertilizer served as a negative control. $LSD_{(T)(0.05)}$ values are indicated for each test crop separately.

Crop	Treatment	Dry grain mass (ton/ha)
Maize	Control	4.9 ± 0.3
	ComCat®	*5.2 ± 0.5
	SS	*5.2 ± 0.4
	$LSD_{(T)(0.05)}$	0.24
Wheat	Control	1.9 ± 0.1
	ComCat®	2.4 ± 0.2
	SS	*2.6 ± 0.1
	$LSD_{(T)(0.05)}$	0.52

* = Data differing significantly from the untreated control according to Tukey Kramers least significant difference (LSD) statistical procedure at P<0.05.

fied further by means of P-TLC. 50 mg of each column fraction dissolved in 50 µl of the mobile phase was loaded in a band and in 10 µl increments onto a separate pre-coated Silica plate (Merck Kieselgel 60 F_{254}; 20 × 20 cm^2, 1 mm thickness) by streaking evenly over the full length of the baseline with the aid of a glass capillary tube. Using a fan, the plate was air dried between increments to concentrate the substances in a small area as possible and, subsequently, developed in a saturated glass chromatography chamber using chloroform: methanol (10:1; v/v) as mobile phase. After observing the plate under UV light (254 and 365 nm) and marking the clearly defined boundaries of individual compounds, the silica containing the compounds were scraped off from the glass plates using a spatula and transferred to separate glass beakers. The compounds were recovered from the Silica by elution with ethanol for compounds with RF-value above 0.5 and methanol for those with RF-values below 0.5 (Mikes and Chalmers, 1979) followed by centrifugation for 5 min at 20,000 g. Only one active compound was recovered from the pooled column fractions and its purity confirmed in an original analytical TLC system. Its chemical structure was elucidated by means of mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

Chemical structure elucidation of the purified compound

The isolated compounds were washed repeatedly with acetone to obtain an acceptable level of purity. Subsequently, the compounds were analyzed by spectroscopic and spectrometric methods [fast atom bombardment (FAB-MS)] and electron impact mass spectrometry (EI-MS)]. Nuclear magnetic resonance spectroscopy (NMR) was performed on a Bruker 300 MHz DRX 300 spectrometer at 296 K (23°C) with tetramethylsilane [$Si(CH_3)_4$; TMS] as the internal standard. The solvents used were either deuteriochloroform ($CDCL_3$) or deuterio-acetone [$(CD_3)_2$ CO] as indicated. Chemical shifts were reported in parts per million (ppm) on the δ-scale and coupling constants are given in Hz. The following abbreviations were used: s = singlet, m = multiplet, d = doublet, t = triplet, dd = doublet of doublets, br = broadened, DEPT = distortionless enhancement by polarization transfer, COSY = correlation spectroscopy, NOESY = nuclear overhauser effect spectroscopy, HMBC = heteronuclear multi-bond coherence, HMQC = heteronuclear multiple quantum coherence, ^{13}C NMR = ^{13}carbon nuclear magnetic resonance, ^{1}H NMR = proton nuclear magnetic resonance, FAB-MS = fast atom bombardment, EI-MS = electron impact. All FAB mass spectra were recorded on a VG 70-70 E double-focusing mass spectrometer and mass determinations were obtained with a Kratos MS-80 mass spectrometer in the double focus electron impact (EI) mode.

Structural elucidation was mainly achieved through NMR spectroscopy (1D and 2D) as well as chemical methods, for example, hydrolysis. The 1D NMR experiments comprised the ^{1}HNMR, ^{13}CNMR and DEPT while the 2D included HMQC and HMBC.

Statistical analysis

Analysis of variance (ANOVA) was performed on the data, using the NC: SAS Institute Inc., Dos Statistical Programme to identify differences between treatments. Tukey-Kramer's least significant difference (LSD) procedure for comparison of means (Steele and Torrie 1980; Mason et al., 1989) was applied to separate means. In all cases $LSD_{(T)(0.05)}$ values are supplied in tables and figures while significant differences are indicated with an asterisk (*).

RESULTS

Yield response of maize and wheat to foliar treatment with a *L. albus* seed suspension (SS) under field conditions

Both ComCat® and SS contributed to significant yield increases in maize after a single foliar spray treatment at the 3 to 5 leaf growth stage, compared to the untreated control (Table 4). Similar results were obtained with wheat, but compared to the untreated control this was only statistically significant (P = 0.05) in the case of SS. For both maize and wheat, the yield response to treatment with SS compared favourably with that of treatment with the commercial bio-stimulant as differences in yield enhancement between the two treatments were non-significant. This as well as previous results obtained by Van der Watt and Pretorius (2011) supplied the rationale for isolating and purifying the active compound(s) involved from SS.

Bio-stimulatory activity of a *L. albus* seed suspension (SS) and a crude extract thereof

As the manufacturers of ComCat® claimed a 0.5 mg ℓ^{-1} concentration to be optimal for bio-stimulation of crop plants and the minimum stimulatory concentration (MSC) of SS was found to be 5.0 mg ℓ^{-1} with both bio-assay procedures, only these concentrations were applied in the extended screening program with different seedlings. All test solutions significantly enhanced the respiration rate of monoculture yeast cells compared to a water control (Table 5). The optimum concentration for ComCat® (0.5 mg ℓ^{-1}) showed an inhibitory effect on the hypocotyl and root growth of Cress seedlings; although, not significantly in the first case (Table 5). In comparison, SS im-

proved the hypocotyl growth of Cress seedlings at the optimum concentration of 5 mg ℓ^{-1}, but also non-significantly (Table 5). However, both the seed suspension (SS) and crude extract thereof significantly enhanced the root growth of Cress seedlings. Subsequently, the screening process was expanded to seeds and seedlings of maize and wheat. At 96 h of incubation, SS and the crude extract as well as ComCat® significantly enhanced coleoptile and root growth of wheat and maize seedlings (Table 5). Similar bio-stimulatory activity by both SS and the crude extract supplied the rationale for purifying the active substance(s) involved.

Isolation, purification and identification of active compounds from a crude *L. albus* seed suspension

Following activity directed liquid-liquid extraction of the seed suspension, it was only ethyl acetate fraction 1 (Figure 1) and the hexane fraction that significantly ($P<0.05$) increased the respiration rate of monoculture yeast cells compared to the water control (Table 6). This compared favourably to that of the commercial product, ComCat®, used as positive control. Significant bio-stimulatory activity was also confirmed in ethyl acetate fraction 1 in terms of root and hypocotyl growth of Cress seedlings (Table 6). Although, ethyl acetate fraction 2 also significantly enhanced root growth in Cress seedlings, this was neither the case for hypocotyl growth nor yeast cell respiration. Subsequently, only ethyl acetate fraction 1 that was found to be significantly active in all bioassays was further fractionated by means of column chromatography. From this, 85 column fractions were obtained that were pooled into 12 combined fractions on grounds of similarities in Q-TLC profiles. Fractions 1 to 3 and fraction 10 showed significant bio-stimulatory activity while fractions 4, 7 and 9 showed marked but non-significant activity in terms of root growth enhancement of Cress seedlings (results not shown). The results revealed a clear pattern in terms of bio-stimulatory activity namely, a large peak represented by column fractions 1 to 4 and a small peak represented by fractions 9 and 10. As the large peak contained the highest amount of recovered dry matter and showed similarities in their Q-TLC profiles, these fractions were combined and subjected to a further purification step using preparative thin layer chromatography (P-TLC).

Activity directed P-TLC separation of compounds contained in the first four combined column fractions yielded one pure compound that was shown to be highly active by using both bio-assays (results not shown). This compound was subjected to nuclear resonance spectroscopy in order to identify the compound and elucidate its structural formula. The ^{1}H NMR and ^{13}C NMR resonances (Table 7) of the purified compound did not reveal aromatic protons. However, 98 aliphatic protons were observed in the ^{1}H NMR spectrum. Assignment of the protons was aided by ^{13}C NMR, HMQC, HMBC and DEPT experiments (results not shown). The results of the ^{13}C NMR, DEPT and HMQC experiments revealed key points that were crucial in the elucidation of the structure. Importantly, the C-H protons resonated only between δ 5.3 and 5.4, the methyl groups at δ 0.9 and the rest of the peaks being methylene protons. The deshielded methyl-lene protons (δ 4.1 to 4.4) and methine proton (δ 5.30) suggests protons attached to oxygen bearing carbons, implicating a glycerol ester type of structure. The olefinic complex multiplet resonating at δ 5.4 was assigned to the aliphatic double bonds (H-9/10 and 12/13), the doublet of doublets between δ 4.1 and 4.3 to H–1' (2H) and 3'(2H) and the deshielded multiplet at δ 5.3 to the tertiary oxomethine (H-2', C-69.3 ppm). Couplings from the methylene protons (δ 4.1 to 4.3) to the deshielded methine (δ 5.3) as observed in the COSY experiment and NOESY (results not shown) associations of the same protons confirmed the glycerol part of the structure. Strong HMBC of H-1'/3' to C-2' and C-1 (carbonyl carbon, C-173.6 ppm) and H-2' to C-1 unambiguously confirmed the presence of the glycerol ester in the structure. The aliphatic double bonds (δ5.35, C-128.2, 128.4, 130.3 and 130.6) showed a strong COSY correlation to the bis-allylic methylene protons (H-11) at δ 2.75 and the mono-allylic protons (H-8 and 14) 2.0, confirming the H-9/10 and H-12/13 *cis* double bonds. The methylene protons (δ 2.3, triplet) were confirmed by the observed strong HMBC they showed to C-1. The rest of the protons were established from the COSY, NOESY and HMBC spectra.

In order to confirm the ester linkage, the compound was hydrolyzed. The compound was refluxed with 0.1 M hydrochloric acid for 1 h and the product extracted with ethyl acetate. With exception of the methylene (δ 4.1 to 4.3) and the methine protons (δ 5.3), the protons in the ^{1}H NMR spectrum of the hydrolyzed product were identical to those in the ^{1}H NMR of the starting material (compound 1). The results indicated that an ester linkage was cleaved and the fatty acid moiety was recovered. The ^{1}H NMR spectrum of the product from the hydrolysis was identical to that of authentic linoleic acid. The molecular structure of $C_{57}H_{98}O_6$ which was in agreement with the calculated molecular mass of 879 confirmed the glycerol ester to be a known C-57 glyceryl trilinoleate (Figure 3). By means of different bio-tests, glyceryl trilinoleate was confirmed to be the active bio-stimulatory compound in seeds of *L. albus* cv Bethuana White. Subsequently, the newly identified glyceryl trilinoleate (SS a.i.) was compared to ComCat® and commercially available pure trilinolein in terms of its effect on the respiration rate of monoculture yeast cells (Figure 2A) as well as on wheat and maize root growth (Figure 2B). The glyceryl trilinoleate (SS a.i.) isolated from *L. albus* cv Bethuana White seeds significantly ($P<0.05$) enhanced the respiration rate of

Table 5. *In vitro* bio-stimulatory activity of a *L. albus* cv Bethuana White seed suspension (SS) and a crude extract thereof measured in terms of respiration rate in monoculture yeast cells as well as seedling growth in wheat, maize and Cress seedlings.

Bioassay	Measured activity	Water	ComCat®	SS powder	SS crude	$LSD_{(T)(0.05)}$
Yeast cells	Respiration rate (cm^3 CO_2 80 min^{-1})	4.4 ± 0.3	10 ± 0	10 ± 0	10 ± 0	1.15*
Wheat seedlings	Coleoptile length (mm)	25.8 ± 1.0	27.2 ± 0.4	28.4 ± 0.5	28.8 ± 0.2	1.78*
	Root length (mm)	73.1 ± 2.0	86.0 ± 0.5	90.4 ± 0.5	87.8 ± 3.4	6.36*
Maize seedlings	Coleoptile length (mm)	31.5 ± 1.0	50.4 ± 1.5	44.5 ± 0.9	43.3 ± 0.7	3.22*
	Root length (mm)	76.0 ± 1.7	89.4 ± 1.2	88.7 ± 1.4	84.5 ± 3.4	6.52*
Cress seedlings	Hypocotyl length (mm)	30.4 ± 4.5	22.5 ± 7	34.9 ± 2.6	34.5 ± 1.8	8.13
	Root length (mm)	57.7 ± 13.9	45.7 ± 17.6	79.6 ± 4.1	78.8 ± 3.5	10.49*

***** = Data differing significantly from the untreated control according to Tukey Kramers least significant difference (LSD) statistical procedure at P<0.05.

Table 6. Activity directed bio-assaying of semi-purified SS fractions obtained by means of liquid-liquid extraction. Respiration rate of monoculture yeast cells as well as coleoptile and root growth of Cress seedlings were used as parameters.

Bioassay	Measured activity	Treatment						
		Water	ComCat®	Ethyl acetate 1	Hexane	$NaHCO_3$	Ethyl acetate 2	$LSD_{(T)(0.05)}$
Yeast cells	Respiration rate (cm^3 CO_2 min^{-1})	59.5±0.7	111.6±2.8*	119.7±0.5*	110.1±0.0*	57.3±1.5	58.1±1.4	4.38
Cress seeds	Hypocotyl length (mm)	31.2±0.1	32.8±2.8	36.6±2.3*	34.5±2.47	35.0±4.1	36.1±0.3*	4.48
	Root length (mm)	61.8±0.1	63.2±1.0	73.3±3.6*	60.6±0.6	58.6±1.9	56.1±5.1	4.59

***** = Data differing significantly from the untreated control according to Tukey Kramers least significant difference (LSD) statistical procedure at P<0.05.

monoculture yeast cells compared to the water control from 40 min of incubation and onwards until the maximum rate of 10 cm^3 CO_2 min^{-1} was reached (Figure 2A).

It also compared favourably with the pure commercially obtained trilinolein and ComCat® that both significantly increased the respiration rate of yeast cells.

Additionally, after 96 h of incubation, the purified glycerol trilinoleate (SS a.i.), commercial trilinolein and ComCat® significantly increased root growth in maize seedlings (Figure 2B), compared to the water control, while no significant differences were observed between the different bio-stimulatory compounds. Wheat root growth was significantly increased by ComCat® (12%) and SS a.i. (25%), compared to the water control, while the commercial trilinolein contributed to a marked, but non-significant increase.

DISCUSSION

According to the study of Schnabl et al. (2001), extractable amounts of plant growth regulating compounds are associated with the plant families Fabaceae and Caryophyllaceae. This supplied the

Table 7. ^{1}H NMR and ^{13}C NMR data of the bio-stimulatory active compound purified from *L. albus* L. seeds.

Proton(s)	$CDCl_3$ 298 K	Carbon	ppm (298 K)
		1	173-
H-2	2.30(t)	2	34.5
H-3	1.60 (m)	3	26.5
H-4-6	1.29 (m)	4-6	22.6-34.7
H-7	1.30 (m)	7	22.6-34.7
H-8	2.00 (m)	8	27.5
H-9	5.35 (m)	9	128.2
H-10	5.35 (m)	10	130.3
H-11	2.80 (m)	11	26.0
H-12	5.35 (m)	12	128.4
H-13	5.35 (m)	13	130.6
H-14	2.00 (m)	14	27.5
H-15	1.30 (overlapped)	15	22.6-34.7
H-16-17	1.29 (overlapped)	16-17	22.6-34.7
CH_3	0.9 (m)	18	14.5
H-1'/3'	4.1-4.3 (dd)	1'/3'	62.5
H-2'	5.30 (m)	2'	69.3

rationale for a comprehensive screening program that commenced in 2002 during which extracts of different South African species from these two families were screened for bio-stimulatory activity in our laboratory (Pretorius et al., 2008). As part of the outcome, we recently reported that a *L. albus* L. cv. Bethuana White (Fabaceae) seed suspension (SS) showed significant *in vitro* bio-stimulatory properties by using different bioassay procedures (Van der Watt and Pretorius, 2011). These included the respiration rate of monoculture yeast cells as well as seed germination and subsequent seedling growth of four different vegetable crops. However, *in vitro* data on the bio-activity of plant extracts is of little use if not verified by *in vivo* data under field conditions. For this reason, and the fact that yield increase is the principle objective of applying bio-stimulants to crops from an agricultural perspective, *in vivo* field trials were included in this study. For this purpose, two economically important row crops, maize and wheat were used in order to assess the application potential of SS in the agricultural industry and to find a rationale for the isolation and identification of the active compounds involved. A commercial bio-stimulant, ComCat® was used as positive control at the optimum application rate of 100 g ha^{-1} for maize and 200 g ha^{-1} for wheat as recommended by the distributors (Agraforum SA, 2010).

In both crops, foliar treatment with SS contributed to significant yield increases, compared to the untreated control while not differing significantly from the yield response of the two crops to treatment with the commercial bio-stimulant. Interestingly, already a decade ago, Barczak (2002) reported that leek plants (*Allium* sp.) sprayed with a crude lupin extract showed marked yield increases ranging from 3 to 28% depending on the species. A report by Przybylak et al. (2005) also confirmed the growth and yield response of greenhouse cultivated paprika treated with a lupin extract after seedling establishment. To date, neither of these and other studies (Cwojdzinski et al., 1989; Wysocki et al., 2001) supplied a mechanistic explanation for the response of plants to treatment with a lupin extract. For this reason, an attempt was made to isolate and identify the active compound(s) involved and contained in a crude *L. albus* seed extract. Standard activity directed chromatographic and bio-assay procedures were employed to isolate and purify the active bio-stimulatory compound(s) from *L. albus* seeds. Initial liquid-liquid extraction of ground seed yielded a highly active ethyl acetate extract. Subsequently, compounds contained in this fraction were separated by means of column chromatography. Four highly active combined column fractions, based on similarities in Q-TLC profiles and Rf-values were recovered. The four active column fractions were pooled and from this a single active compound was isolated by means of preparative thin layer chromatography. The purity of the compound was confirmed by means of Q-TLC and its bio-activity in terms of enhanced respiration rate in monoculture yeast cells as well as root growth enhancement in maize and wheat seedlings.

Subsequently, the pure active compound was subjected to nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry to elucidate its structure and identify the compound by name. The different approaches assisted in identifying the active compound as glyceryl trilinoleate, a tri-ester with three fatty acid moieties, linoleic acid,

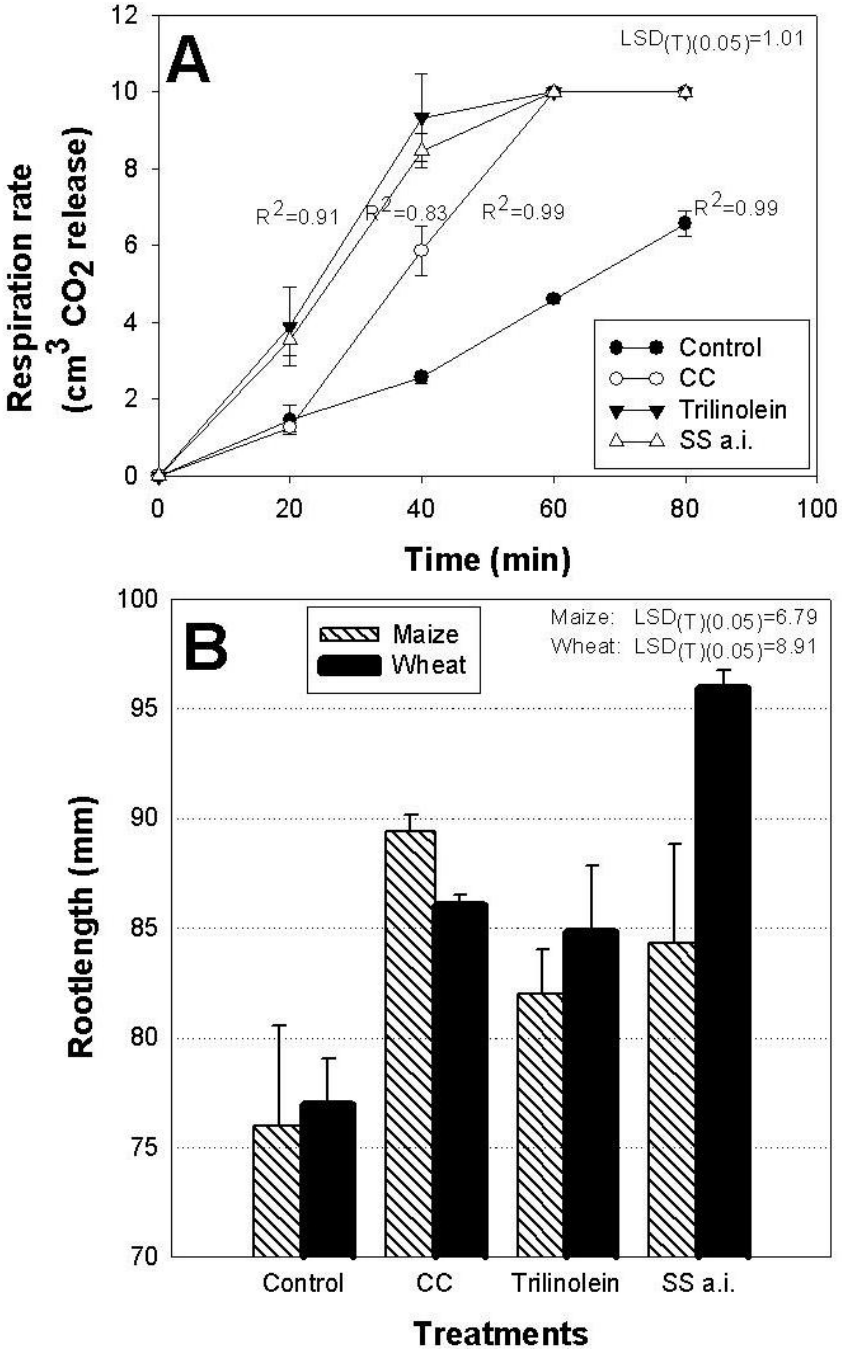

Figure 2. The effect of glyceryl trilinoleate (SS a.i.) isolated and purified from *L. albus* seeds, at a concentration of 0.5 mg L^{-1}, on A) the respiration rate of monoculture yeast cells and B) seedling growth of wheat and maize. Water served as a negative control while a commercial bio-stimulant, ComCat® (CC), and commercially available pure trilinolein ($C_{57}H_{98}O_6$) served as positive controls. Statistical significance is indicated by calculated $LSD_{(T)(0.05)}$ values in the graphs.

attached to glycerol. The bio-activity of the compound was confirmed and compared favourably with that of commercially available pure trilinolein. This is in concert with a report by Han et al. (2007) that tested fatty acids for their plant growth stimulatory activity in the elongation of rape hypocotyls as well as wheat coleoptiles. Further,

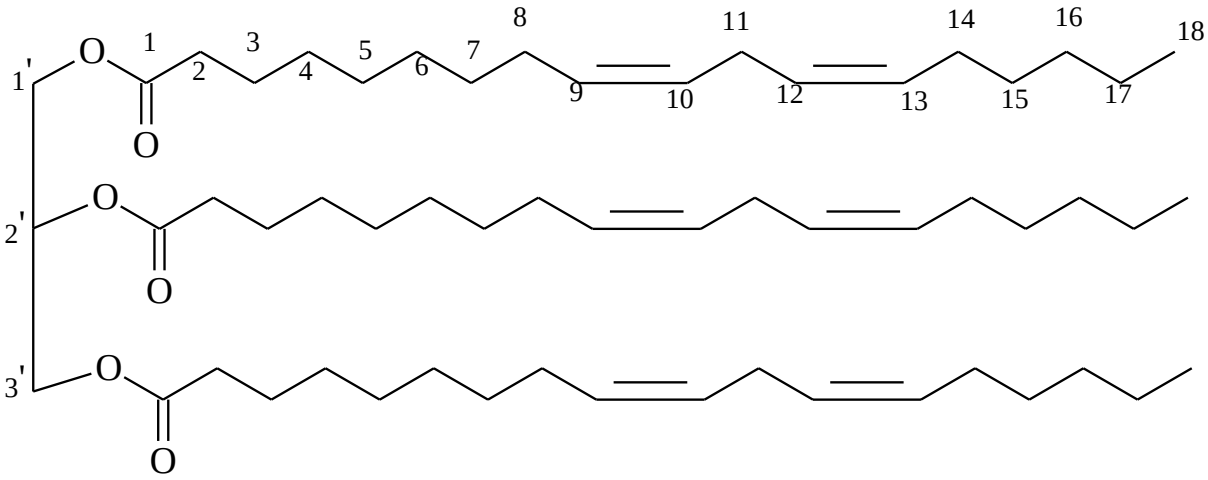

Figure 3. Chemical structure of a bio-stimulatory compound isolated and purified from *L*. *albus* seed and identified as C-57 glyceryl trilinoleate ($C_{57}H_{98}O_6$).

a number of reports pointed towards plant growth regulatory activity contained in plant sterols and fatty acids (Gong et al., 1999; Sheng et al., 2000; Padmapriya and Chezhiyan, 2002; Park et al., 2002; Edqvist and Farbos, 2003; Jensen et al., 2004). For example, the sterol estrone increases growth of root tips and pollen tubes of *Rumex tenuifolius* (Seigler, 1998); while β-sitosterol initiate flower bud formation in *Chrysanthemum* species (Padmapriya and Chezhiyan, 2002). Edqvist and Farbos (2003) reported on the high levels of the epoxidated fatty acid vernolic acid in the endosperm of *Euphorbia lagascae* Spreng seeds that play a role during seed germination after being mobilized and oxidized. Weber (2002) reported a few fatty acids that are involved in many plant developmental anomalies such as reduced apical dominance and delayed senescence.

Despite its growth promoting characteristics, fatty acids have also been shown to be involved in defence mechanisms in plants and to promote chlorophyll synthesis. More specifically, application of fatty acids to *Arabidopsis* mutants showed that they play an essential role in maintaining photosynthetic activity and may act as a second source of precursors for the synthesis of chloroplast glycerolipids (Routaboul et al., 2000). Application of a 0.1% sucrose fatty acid ester (SFE) during flowering and pod filling in soybeans increases the activity of superoxide dismutase by 41% and chlorophyll b content by 58% reaching its maximum 4 to 5 days after application (Ye et al., 2000). These trienoic fatty acids (TA) are activated in response to various environmental stimuli for example temperature stress (Murakami et al., 2000). Numerous studies reported fatty acids that are involved in systemic acquired resistance against diverse pathogens activating defence-related responses (Wink, 2000; Nandi et al., 2004). These fatty acids diffuse through the cytosol and become incorporated in the phospholipids of the plasma membrane while others activate NADPH oxidase (Yaeno et al., 2004). NADPH oxidase in turn causes an oxidative burst, preventing spread of pathogens after infection (Torres et al., 2002). Gong et al. (1999) reported that linoleic acid exogenously applied to barley seedlings during salt stress increases K^+ absorption and translocation, increases the phospholipid content in the tonoplast and increases the activities of H^+-adenosine triphosphatase (ATPase) and H^+-pyrophos-phatase (PPase). The latter treatment also decreases the leakage of electrolytes and Na^+-absorption in turn influencing the translocation of Na^+ to the shoots.

Fatty acids can additionally act as substrates for the lipoxygenase (LOX) enzyme in plants that catalyses its oxidation to hydroperoxide fatty acids which, in turn, act as precursors of jasmonic acid and related compounds (Kolomiets et al., 2001). The authors reported that LOX is involved with physiological processes such as growth, senescence and stress-related responses and that suppression of LOX activity results in reduced tuber yield, decreased tuber size and disruption of tuber formation in potatoes. Moreover, Eastmond et al. (2000) suggested that lipids can be used as a source of carbon for respiretion in germinating oilseeds and that the product of fatty acid catabolism, Acetyl Co-enzyme A, can pass from the peroxisome to the mitochondrion independent of the glyoxylate cycle. However, according to the authors, an additional anaplerotic source of carbon is required during seedling establishment due to an increased demand and the glyoxylate cycle can act as this additional source. In summary, by means of *in vitro* bio-assays, it was confirmed in this study that glyceryl trilinoleate was the active compound responsible for the bio-stimulatory activity contained in *L. albus* seeds. Moreover, similar results

obtained with commercially available trilinolein as well as numerous reports in literature on the bio-stimulatory properties of fatty acids, served as further validation of obtained results. However, the growth response of wheat seedlings to treatment with the purified active compound from *L. albus* seeds seemed to be slightly more sensitive than that of maize seedlings indicating that other crops might also react differently. This warrants a further and extended field study which should include treatment with the seed suspension (SS) as well as one or more commercial bio-stimulants.

Treatment with SS is essential to verify whether synergism between compounds in a more crude form of *L. albus* seed is a possibility and treatment with positive controls is to assess the commercial potential of glyceryl trilinoeate from an agricultural perspective. This extended trial program is currently being followed over three seasons together with attempts to elucidate the mechanism of action involved. Finally, after all the necessary data has been acquired, the possibility for developing a natural product with application potential in the agricultural Industry is not excluded.

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