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Physiological Effect of Some Antioxidants on Flax Plant (*Linum usitatissimum* L.)

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Abstract: Two pot experiments were conducted in the screen of the National Research Centre during two successive seasons ((2008/2009 and 2009/2010) to study the physiological effect of the antioxidants putrescine (40, 80 and 120 mg/l), α-tocopherol (100, 200 and 300 mg/l) and stigmasterol (100, 150 and 200 mg/l) on flax plant. The obtained results indicated that foliar application of putrescine, stigmasterol or α-tocopherol significantly affected growth criteria and seed yield of flax plants. Photosynthetic pigments and total protein%, total phenols in the leaves at 60 days after sowing as well as oil% in the seeds. On the other hand, all treatments decreased the activity of polyphenol oxidase in leaves. The amino acid proline was found to be the main amino acid and Linolenic acid was found to be the main fatty acid in the seeds of all treatments under study.

Key words: Flax *(Linum usitatissimum)* · Putrescine · Stigmasterol · α-tocopherol · Chemical constituents

INTRODUCTION

Flax *(Linum usitatissimum* L.) seeds are widely used medicinally. They are used as emollient, demulcent and pectoral. The crushed seeds or linseed meal make a very useful poultice, either alone or with mustard. In ulceration and superficial or deep-seated inflammation a linseed poultice allays irritation and pain and promotes supporation [1]. Their constituents include 30-40 per cent of fatty acids (linseed oil) with esters of linoleic acid (60 per cent), linolenic acid (20 per cent), stearic acid (8 per cent) and oleic acid; also mucilage, proteins and cyanogenic glycosides. The oil also is important in the manufacture of paints; soap and printer's ink [2]. Polyamines are low-molecular weight polycations, which are involved in nearly all developmental processes and also in the regulation of growth and stress, probably by binding to negatively charged macromolecules [3-5].

Stigmasterol is found as a free or compound in the cell. They are structural components of the lipid core of cell membrane and being precursor of numerous secondary metabolites including plant steroid hormones [6]. Much less the biological function of steryl conjugates such as fatty acid or glucoside esters and steryl acyl glucoside were as a sterol storage forms. It has been shown that membrane sterol composition seems to have an effect on the activity of H^+-ATPases in plant [7]. Brassinosteroids increased shoot and root growth, increased levels of DNA, RNA, protein, carbohydrates and increased yield and seed lipid contents in groundnuts [8]. Xian-He *et al.* [9] indicated that sterols play a crucial role in plant cell division, embryogenesis and development. Both sterols and brassinosteroids activate regulators of plant development and gene expression. Similar to brassinosteroids, both typical (sitosterol and stigmasterol) affect the expression of genes involved in cell expansion and cell division. Wilen *et al.*[10] indicated that brassinosteroids increase heat tolerance in brome grass cells and induce the synthesis of heat-stable polypeptides as well as the accumulation of heat shock protein 90KDa that may have a role in conferring heat tolerance.

Alpha-tocopherol (vitamin E) is low molecular weight lipophilic antioxidant which mainly protect membrane from oxidative damage [11]. Zhang *et al.* [12] reported a positive correlation between α-tocopherol and shoot or root growth in two grass species grown under drought conditions. Tocopherols were reported to function in relation to their antioxidant properties being prominent in protection of polysaturated fatty acids from lipid peroxidation [13]. Recently, tocopherol had an antioxigenic property when added to green lubricating oil as rapeseed oil [14].

The aim of the present study was to reveal the best level to apply of putrescine, stigmasterol and α-tocopherol which could improve growth, yield, chemical constituents and oil production of flax (*Linum usitatissimum* L.) plant.

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MATERIALS AND METHODS

Two pot experiments were carried out during two successive seasons of (2008/2009 and 2009/2010) at the screen of National Research Centre, Dokki, Giza, Egypt. Seeds of flax (*Linum usitatissimum* L., var. Sakha-2) were secured from Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt. Seeds were sown in 30 cm diameter earth ware pots on 30th November, in the first and second seasons, respectively. Each pot contained 8 kg loamy clay soil. At 45 days after planting, plants were sprayed with different concentrations of the antioxidants putrescine (40, 80 and 120 mg/l), α-tocopherol (100, 200 and 300 mg/l) or stigmasterol (100, 150 and 200 mg/l). All used antioxidants were products of Sigma Co. Treatments were distributed in complete randomized design with three replications comprised with three pots for each replicate. Each pot received equal and adequate amounts of water and fertilizers. Phosphorous as calcium superphosphate (15.5% P_2O_5) was added to the soil before sowing at a rate of 4.0 g / pot. Three grams of nitrogen as ammonium sulphate (20.5% N) was applied in three portions (one g for each) with two weeks intervals started 30 days after sowing, also, potassium sulphate (48% K_2O) at a rate of 2g /pot was added as soil application. Other agricultural processes were performed according to normal practice. At 60 days after planting (DAP) a representative sample from each treatment was taken to determine some growth criteria. Also at the same growth stage, photosynthetic pigments were determined in the leaves according to von Wettstein [15]. Total soluble phenols were determined in the leaves according to the method described by Singleton *et al.* [16]. Polyphenol oxidase activity was assayed in the leaves as described by Kumar and Khan [17].Total protein was determined in the seeds at harvest using the method of Bradford [18]. Total amino acids were extracted and estimated according to the method described by Steven *et al.* [19]. The HPLC apparatus used for amino acid estimation is Spectro-Physics Analytical, Inc. A0099-600 with spectra focus optical scanning detector and spectra system UV 2000 detector and ultrasphere C18 Beckman column. Analysis was carried out using a gradient of Pico-Tag solvent A and B at 40°C and a flow rate 1ml/1min. Detection of the separated PICO-tag amino acids was done at 254nm wavelength. Before injecting the sample, the illustration was calibrated by two injections of the standards. Fixed oil extraction and fatty acids composition were estimated according to A.O.A.C. [20].

Conditions of Fatty Acid Methyl Ester on GC

Instrument: agilent Technologies 6890 N Network GC system.

Oven: Initial temp. 70°C, initial time 2 min, Rate 8, final temp 240°C, final time 25 min. Inlet temp. 250°C, Detector temp. 300°C (FID), Flow 1-5 ml/min.

Column: Capillary Column HP-5 5% phenyl methyl siloxane, length 30 m, diameter 320 μm, film thickness 0.25 μm. Carrier gas N_2 30 ml/min, H_2 30 ml/min, Air 300 ml/min.

Data obtained were subjected to standard analysis of variance procedure. The values of LSD were obtained whenever F values were significant at 5% level as reported by Snedecor and Cochran [21]. Combined analysis was made from two growing seasons hence the results of two seasons followed similar trend.

RESULTS AND DISCUSSION

Growth and Yield Components: Data presented in Tables 1 and 2 indicated that foliar application of putrescine to flax plants significantly increased plant height at 60 DAP, yield and technical length / plant of flax plant, especially in plants treated with 120 mg/l putrescine. Fresh and dry weights / plant, number of capsules / plant, number of fruiting zones / plant, biological and straw yields / plant followed the same trend. On the other hand, number of branches / plant, weight of capsules / plant and weight of 1000 seeds were not significantly affected.

In this connection, high levels of free polyamines have been reported to influence growth by cell division and low levels with cell expansion [22]. These basic molecules, which are positively charged at physiological

Table 1: Effect of putrescine, stigmasterol and α-tocopherol on vegetative growth of flax plant at 60 DAP.

Treatments (mg/l)	Plant height (cm)	Fresh wight (g/plant)	Dry weight (g/plant)	No of branches/ plant
Control	42.33	2.67	0.83	3.00
P40	47.33	3.96	0.92	3.00
P80	51.00	3.67	0.92	3.00
P120	54.00	4.08	0.93	3.00
SS100	46.33	3.80	1.02	2.67
SS150	44.67	2.73	0.81	3.00
SS200	46.67	3.89	1.03	4.00
α-tocopherol 100	46.33	3.45	1.15	3.67
α-tocopherol 200	52.50	3.98	1.33	4.00
α-tocopherol 300	47.67	3.60	1.11	3.00
LSD (5%)	4.89	0.76	0.24	N.S.

P: putrescine, SS: stigmasterol

Table 2: Effect of putrescine, stigmasterol and α-tocopherol on yield of flax plant (harvest stage).

Treatments (mg/l)	Plant height (cm)	Technical length (cm)	No. of capsules / plant	Weight of capsules / plant	Number of fruiting zones / plant	1000 seeds (g)	Biological yield (g/plant)	Straw yield (g/plant)
Control	62.89	53.89	8.11	0.62	3.33	6.36	1.50	0.89
P40	69.78	59.78	8.24	0.72	4.78	6.56	2.41	1.71
P80	69.89	60.33	9.44	0.79	4.33	6.57	2.51	1.75
P120	75.22	65.67	9.89	0.81	5.33	6.74	3.32	2.57
SS100	67.89	58.56	9.11	0.69	3.44	6.59	2.29	1.71
SS150	72.22	63.22	9.89	0.78	4.67	7.00	2.34	2.10
SS200	73.22	63.78	10.33	0.85	6.00	7.12	2.58	1.78
α-tocopherol 100	73.44	64.22	9.89	0.70	5.44	6.71	3.51	2.90
α-tocopherol 200	74.45	64.44	10.67	0.80	6.56	6.73	4.20	3.50
α-tocopherol 300	71.89	60.78	9.55	0.69	5.22	6.58	3.08	2.45
LSD (5%)	2.19	3.56	0.92	N.S.	0.59	N.S.	0.46	0.64

pH, are ubiquitous in nature and because of the positive charge; polyamines are known to bind to negatively charged molecules, e.g. nucleic acids, acidic phospholipids and various types of proteins. Martin-Tanguy [23] also reported that polyamines occur in plants in free form, bound electrostatically to negatively charged molecules and conjugated to small molecules and proteins. Talaat *et al.* [24] also reported that exogenous application of putrescine on periwinkle transplants considerably increased plant growth at successive developmental stages. The effect was more pronounced with 10^{-3} M putrescine.

Data presented in Tables 1and 2 also indicated that foliar application of stigmasterol to flax plants significantly increased plant height, technical length/plant, fresh and dry weights of plants. The highest values were obtained in plants treated with 200 mg/l stigmasterol. Fresh and dry weight/plant, number of capsules / plant, number of fruiting zones / plant, biological and straw yields / plant followed the same trend. On the other hand, number of branches / plant, weight of capsules / plant and weight of 1000 seeds were not significantly affected. In this concern, Ayad *et al.* [25] reported that treatment of geranium plants with stigmasterol significantly promoted vegetative growth, especially at cutting (III).

Data presented in Tables 1 and 2 also indicated that α-tocopherol significantly increased plant height at vegetative growth stage, harvest stage and technical length / plant of flax plant, especially in plants treated with 200 mg/l α-tocopherol. Fresh and dry weights of plant, number of capsules / plant, number of fruiting zones / plant, biological and straw yields / plant followed the same trend. On the other hand, number of branches / plant, weight of capsules and weight of 1000 seeds were not significantly affected. Numerous reports are available where the attempt has been made to reduce oxidative stress in plants by exogenous application of tocopherols via the foliage of plants [26]. Plant cells can be protected against oxidant stress by various radical-scavenging systems, including low-molecular-weight antioxidants such as ascorbate, glutathione, α-tocopherol and carotenoids, as well as by antioxidant enzymes such as superoxide dismutases, peroxidases and glutathione reductases [27]. Protection against phytotoxic peroxidation processes in lipophilic environments may be achieved by antioxidants, like α-Tocopherol (Vitamin E), which is assumed to be the most effective radical-chain-breaking substance in the membranes. Therefore, many attempts have been made to reduce oxidative stress in plants by exogenous application of this vitamin [26].

Chemical Constituents: Data presented in Table 3 indicated that treatment of flax plants with putrescine resulted in a significant increase in photosynthetic pigments. The best results were obtained in plants treated with 120 mg/l putrescine. Total proteins%, total fixed oil% and total phenols% followed the same trend. Polyphenol oxidase activity decreased significantly as a result of putrescine treatments. In this concern, Martin-Tanguy [23] reported that polyamines occur in plants in free form, bound electrostatically to negatively charged molecules and conjugated to small molecules and proteins. Similar results were obtained by Youssef *et al.* [28]. In this respect, Talaat *et al.* [24] reported that exogenous application of putrescine on periwinkle transplants considerably increased photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids). Also soluble and total insoluble sugars, total proteins and total alkaloids in the leaves of periwinkle plants were increased as a result of application of putrescine, more so with 10^{-3} M.

Table 3: Effect of putrescine, stigmasterol and α-tocopherol on chemical constituents of flax plant

	Vegetative growth stage						Harvest stage	
Treatment (mg/l)	Chl. a	Chl. b	Carotenoids	Total chl.	Phenols (%)	PPOase activity	Proteins (%)	Oil (%)
Control	1.04	0.63	0.43	1.67	0.88	0.56	24.28	39.86
P40	1.61	0.90	0.69	2.51	0.93	0.21	24.72	40.45
P80	1.59	0.85	0.76	2.44	1.17	0.15	26.84	40.75
P120	1.67	0.85	0.78	2.52	1.64	0.14	28.19	41.39
SS100	1.81	0.93	0.84	2.70	1.43	0.38	25.46	41.29
SS150	0.91	0.61	0.43	1.52	1.78	0.35	28.66	42.02
SS200	1.79	1.08	0.77	2.87	1.81	0.30	30.20	42.52
α -tocopherol 100	1.72	0.89	0.77	2.61	1.11	0.49	24.83	40.08
α -tocopherol 200	2.37	1.39	0.96	3.53	1.26	0.35	26.39	42.43
α -tocopherol 300	2.07	1.20	0.95	3.27	1.25	0.38	25.02	41.11
LSD (5%)	0.24	0.34	0.16	0.65	0.02	0.01	0.17	0.48

Application of stigmasterol to flax plants significantly increased photosynthetic pigments, total proteins%, total oils% and total phenols%, especially in plants treated with 200 mg/l stigmasterol. On the other hand, polyphenol oxidase activity decreased significantly as a result of stigmasterol treatments.

In this concern, Abdel Wahed *et al.* [29] reported that spermidine treatments, except at 100 mg/1, significantly increased phenolic content of chamomile leaves compared to the control. They also found that application of stigmasterol insignificantly increased phenols, except at 75 mg/l showed significant increment. Spermidine treatment (at 25 mg/1) was more effective than the stigmasterol one. It could derive from sugars, free amino acids, phenolic compounds and essential oil. That might be due to the accumulation of high phenolic compounds content in the plant tissue. Moreover, sitosterol increased the total number of the phenolic compounds in rice plant during developmental stages [30]. Application of 200 mg/l α-tocopherol significantly increased photosynthetic pigments in flax leaves. Total proteins%, total fixed oil% and total phenols% followed the same trend. Polyphenol oxidase activity was decreased significantly as a result of α-tocopherol application. In this concern, Zhang *et al.* [12] showed positive correlation between α-tocopherol and shoot or root growth in the two grass species of tall fescue and creeping bentgrass. El-Bassiouny *et al.* [31] also reported that foliar spray with α-tocopherol on faba bean plants induced increments in growth parameters, yield components, chlorophyll a, chlorophyll b and carotenoids contents. Alpha-tocopherol (α-T, vitamin E) helps to maintain the integrity of the photosynthetic membranes under oxidative stress [32]. Tocopherols are a group of compounds synthesized only by photosynthetic organisms. The best characterized and probably most important function of tocopherols is to act as recyclable chain reaction terminators of polyunsaturated fatty acid free radicals generated by lipid oxidation. From a biosynthetic perspective, tocopherols are members of a large, multifunctional family of lipid soluble compounds called prenylquinones that also include tocotrienols, plastoquinones and phylloquinones (vitamin K_1). Tocopherols are thought to be important for free radical scavenging and protection from oxidative stress [33]. In plants, tocopherols are presumed to function as membrane-associated antioxidants and as structural components of membranes, although evidence supporting these roles are limited [34]. Tocopherols are believed to protect chloroplast membranes from photooxidation and help to provide an optimal environment for the photosynthetic machinery [32]. Many of the proposed tocopherol functions in animals and plants are related to their antioxidant properties, the most prominent of which is protection of polyunsaturated fatty acids from lipid peroxidation by quenching and scavenging various reactive oxygen species (ROS) including singlet oxygen, superoxide radicals and alkyl peroxy radicals [32]. In plants, tocopherol levels and composition vary in different tissues and fluctuate during development and in response to abiotic stresses. Significant increases in leaf α-tocopherol levels are observed during aging and senescing of plants [35, 36], possibly to protect cellular components from increased oxidative stress [36]. Enhanced tocopherol accumulation also occurs in response to a variety of abiotic stresses including high light, drought, salt and cold and may provide an additional line of protection from oxidative damage [32, 37]. Regulation of tocopherol biosynthesis in

Table 4: Effect of putrescine, stigmasterol and α-tocopherol on amino acids composition (mg/g) in flax seeds

Amino acids mg/g protein	Control	Putrescine (120 mg/l)	Stigmasterol (200 mg/l)	α-tocopherol (200 mg/l)
Aspartine	2.98	3.54	3.11	3.14
Threonine	1.46	1.11	1.34	0.12
Serine	1.71	3.19	1.61	1.06
Glutamine	5.55	--	6.64	8.28
Proline	30.00	29.62	28.75	20.91
Glycine	1.26	1.42	1.30	1.35
Alanine	2.02	2.14	1.99	1.82
Cystine	5.33	5.57	5.18	5.10
Valine	0.03	--	0.36	--
Methionine	0.74	0.60	0.80	0.55
Isoleucine	1.69	1.75	1.59	1.48
Leucine	2.43	2.56	2.36	2.22
Tyrosine	1.45	1.49	1.44	1.19
Phenylalanine	2.38	2.42	2.23	2.02
Hisidine	1.45	1.49	1.53	1.18
Lysine	1.63	1.67	1.63	1.49
Argenine	4.80	4.80	4.22	4.49

Table 5: Effect of putrescine, stigmasterol and α-tocopherol on fatty acids composition (mg/g) of flax oil

Fatty acids mg/g fixed oil	Control	Putrescine (120 mg/l)	Stigmasterol (200 mg/l)	α-tocopherol (200 mg/l)
Lauric (C12:0)	1.05	1.17	1.16	1.27
Myristic (C14:0)	0.93	1.24	1.08	3.30
Palmitic (C16:0)	4.51	2.27	2.91	3.32
Stearic (C18:0)	3.21	2.58	1.26	3.37
Total saturated	9.70	7.26	6.41	11.26
Oleic (C18:1)	10.71	1.43	5.85	8.76
Lenoleic (C18:2)	5.48	2.45	7.24	3.54
Linolenic (C18:3)	12.19	4.62	9.31	11.69
Behenic (C22:0)	2.65	2.23	1.24	4.22
Lignocenic (C24:0)	5.37	2.65	4.46	3.79
Total unsaturated	36.40	13.38	28.10	32.00
Total Identified	46.11	20.65	34.51	43.25
T.Uns./TS.*	3.75	1.84	4.38	2.84

*T. Uns. / T.S. = (total unsaturated / total saturated fatty acids ratio)

senescing and stressed plants may occur at multiple steps of the pathway. The enzyme *p*-hydroxyphenyl pyruvate dioxygenase (HPPD) activity limits tocopherol synthesis in non-stressed *Arabidopsis* plants [38].

Application of putrscine on maize plants retards senescence by inhibition of ethylene biosynthesis and increased fresh weight than control [39]. Talaat *et al.* [24] reported that photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids), soluble and total insoluble sugars, total proteins and total alkaloids in the leaves of periwinkle plants were increased as a result of application of putrescine, more so with 10^{-3} M. Perez-Amador *et al.* [3] reported that putrescine reached the maximum level 6 to 10 h after damaging in locally wounded leaves and decreased to basal levels 24 h after wounding. A similar variation in putrescine level was detected in systemic leaves. It was also observed a transient decrease in the level of free spermine, which is coincident with the increase in putrescine after wounding.

Data presented in Table 4 indicated that 17 amino acids were detected in flax seeds. The main amino acid in untreated (control) plants was proline followed by glutamine and cystine. Application of 120 mg/l putrescine resulted in slight decrease in proline content. Meanwhile, glutamine and valine were not detected in this treatment. On the other hand, proline content in plants treated with 200 mg/l stigmasterol was more decreased than the control plants and a slight increase in glutamine content was noticed. Treatment of flax plants with 200 mg/l α-tocopherol resulted in a remarkable decrease in proline content and an increase in glutamine content. The fact that polyamines can activate protein synthesis [40], who suggested that they are activators of this process early in germination. Polyamines (i.e. putrescine, spermine and spermidine) are low-molecular weight polycations, which are involved in the regulation of growth and stress, probably by binding to negatively charged macromolecules [3-5].

Abdel Wahed and Gamal El-Din [29] reported that spraying the chamomile plants with spermidine or stigmasterol significantly decreased total free amino acids content compared to the control (except for the spermidine treatment at 75 mg/1). Free amino acids content decreased under both bioregulators application, especially at the concentration of 100 mg/1. Decrease rates ranged from 5.0% to 51.1% and 19.9% to 66.7%, respectively. It could be due to the bioregulator's effect on translocation processes from leaves to flowers, linking or converting to other plant biosubstances. In this respect, polyamines including spermine and spermidine, linked with particular proteins [27]. That was one of the supposed mechanisms through which polyamine could regulate cell division and related growth processes. In addition, decrease in free amino acids content might be indirectly due to the brassinosteroid effect on enzymatic activities of membrane potential DNA, RNA and protein synthesis [30].

The relative percentages of fatty acids extracted from flax seeds are presented in Table 5. Nine fatty acids were identified, major fatty acids (more than 10%), minor fatty acids (1-10%) and traces (less than 1%). Total saturated fatty acids ranged from 6.41%-11.26% and total unsaturated fatty acids ranged from 13.39%-36.40%.

The main fatty acid in untreated (control) plants was linolenic acid (12.19%) followed by oleic acid (10.71%) and linolenic acid (5.48%). Application of putrescine (120 mg/l) to flax plants resulted in remarkable decreases in the content of these fatty acids. Linolenic acid content was 4.62%, lignocenic acid (2.64%) followed by stearic acid (2.45%). In flax seeds resulted from plants treated with stigmasterol (200 mg/l) linolenic acid content was 9.31%, linoleic acid (7.24%) and oleic acid (5.85%),while in plants treated with 200 mg/l α-tocopherol linolenic acid content was 11.69%, oleic acid was 8.76% followed by behenic acid (4.22%).

Total unsaturated / total saturated fatty acids ratio ranged from 1.84 - 4.38. Foliar application of putrescine at 120 mg/l resulted in the lowest total unsaturated/total saturated ratio of fatty acids (1.84), while foliar spray with 200 mg/l stigmasterol caused the highest increase in this ratio compared to other treatments (Table 5). In this concern, Yousef *et al.* [41] reported that α-tocopherol improved picual olive total fatty acid composition as unsaturated fatty acid (palmitic, palmitoleic, oleic, linoleic and linolenic acids) in comparison with the control. Youssef *et al.* [36] reported that the highest palmitic acid content in *Matthiola incana* plants was obtained in plants treated with 250 mg/l putrescine.

CONCLUSION

From the above mentioned data, it could be concluded that the antioxidants, putrescine, stigmasterol and α-tocopherol, might play a role in plant phytochemical mechanisms through affecting the metabolism of proteins, fixed oil, phenols and polyphenol oxidase activity, but further studies are needed to learn more about these mechanisms.

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