Gesunde Pflanzen (2017) 69:185–196
https://doi.org/10.1007/s10343-017-0404-x

ORIGINAL ARTICLE

Protective Role of a methanolic Extract of Spinach (*Spinacia oleracea* L.) Against Adverse Effects of UV-C Irradiation on Fenugreek (*Trigonella foenum-graecum* L.) Seedlings

Abdel Haleem Mohammed Ahmed Mohammed[1] · Samia Ageeb Akladious[1]

Received: 17 August 2017 / Accepted: 20 September 2017 / Published online: 24 October 2017

Abstract Photosynthesis is an important process for plants in which plants utilize the energy from sunlight to manufacture and produce all their own food. Although UV-C damage is not physiologically relevant for plants growing in the sun, short-wavelength (UV-C) radiation from germicidal lamps has been used to study cell damage in animals as well as in plants. This study provides a review of the effects of a methanolic extract of spinach (SE) as a natural powerful antioxidants used at two concentrations (25 and 50 ppm) on fenugreek seedlings grown under UV-C stress conditions. The results revealed that exposure of fenugreek seedlings to low UV-C period (30 min) increased growth criteria of the produced seedlings. In addition, the contents of chlorophyll, total pigments, total protein, free amino acids, non-enzymatic (carotenoids, total phenol, flavonoids, α-tocopherol & ascorbic acid contents) and enzymatic antioxidants were increased at this period. By increasing exposure periods of UV-C radiation, the growth criteria and chemical constitutes of the seedlings were decreased. Application of lower concentration of SE (25 ppm) improve the growth of the seedlings at 30-min UV-C and was able to protect seedlings from high periods of UV-C irradiation by increasing all measured parameters except CAT. These increases might be an adaptive mechanism to minimize the adverse effects of longer exposure of UV-C radiation. Moreover, treating the irradiated seedlings with spinach extract led to an obvious alteration in gene expression including synthesis of some proteins bands and disappearance of other protein sets of the protein profile.

Keywords Spinach extract · UV-C irradiation · Antioxidant activity · Fenugreek seedlings-Enzymatic & non-enzymatic mechanisms

Die protektive Rolle eines methanolischen Spinatextraktes (*Spinacia oleracea* L.) gegen Nebenwirkungen der UV-C-Bestrahlung auf Bockhornkleesprosse (*Trigonella foenum-graecum* L.).

Zusammenfassung Die Photosynthese ist ein wichtiger Prozess für Pflanzen, bei dem sie die Sonnenenergie zum Aufbau und zur Erzeugung ihrer lebensnotwendigen Stoffe benutzen. Obwohl die UV-C-Schäden physiologisch für die in der Sonne gewachsenen Pflanzen nicht relevant sind, wurde die Kurzwellenradiation (UV-C) aus Ultraviolett-Lampen verwendet, um Zellenschäden bei Tieren und Pflanzen zu untersuchen.

Diese Studie setzt sich mit den Resultaten über die Wirkung des Spinatextraktes (SE) als natürliches, starke Antioxidationsmittel in zwei Konzentrationen (25 und 50 ppm) auf die Bockhornkleesprossen, die unter belastenden UV-C-Bestrahlungen aufgewachsen sind, auseinander. Die Ergebnisse zeigen, dass die Bestrahlung der Bockhornkleesprosse mit UV-C für eine kurze Zeitdauer (30 min) die Wachstumsmerkmale der produzierten Sprosse erweitert. Außerdem erhöht sich der Gehalt an Chlorophyll, Gesamtpigmenten, Gesamtprotein, freien Aminosäuren, nicht-enzymatischen (Karotinide, Gesamtphenole, Flavonoide, Alpha-Tocopherol und Ascorbicsäure) und enzymatischen Antioxidationsmitteln in dieser Periode. Bei einer Verlängerung der Be-

✉ Samia Ageeb Akladious
samiapola@yahoo.com

[1] Biological and Geological Sciences Department, Faculty of Education, Ain Shams University El Makres St. Roxy, 1575, Cairo, Egypt

strahlungszeit mit UV-C verringern sich die Wachstumsmerkmale und die chemischen Bestandteile der Sprosse. Die Verwendung der niedrigen SE-Konzentration (25 ppm) verbessert die Wachstumsmerkmale der Sprosse bei 30-Minuten-UV-C-Bestrahlung und schützt die Sprosse vor langen Perioden der UV-C-Irradiation. Das zeigt sich an der Steigerung aller Messparameter außer CAT. Diese Steigerung könnte ein Kompensationsmechanismus zur Reduzierung der schlechten Einflüsse der langen UV-C-Radiation sein. Außerdem führt die Behandlung der bestrahlten Sprosse mit dem Spinatextrakt zu einer klaren Veränderung in der Genexpression: zum einen in der Synthese sowie sowie im Verschwinden Proteingruppen in dem Proteinprofil.

Schlüsselwörter Spinatextrakt · UV-C-Antioxidationsprozess · Bockhornkleesprosse · Enzymatischer und nicht-enzymatischer Mechanismus

Introduction

Plants use sunlight for photosynthesis process and, as a result, are exposed to the ultraviolet radiation that is present in sunlight. Ultraviolet radiation (UV) is a type of solar radiation with wavelengths between 100 and 400 nm. It ends where the colours of rainbow start. Solar UV radiation can be divided into three types, UV-A (315 to 400 nm), UV-B (280 to 315 nm) and UV-C (100 to 280 nm) spectral ranges. AI1 types of UV radiation are known to damage various plant processes (Rahimzadeh et al. 2011). Because of short wavelength and high energy of UV-C radiation, it would cause severe damage to living cells in both plants and animals. During irradiation the high energy radiation pass through the matter causing ionizing or electric or magnetic disturbances that affect the internal structure or matter of plants. UV-C photons are highly energetic, thus high levels of damage can be created quickly. Such damage can be classified into two categories: damage to DNA and damage to physiological processes. Different studies have shown that UV-C induces diverse growth responses in plants, which in turn has strong negative effects on plant morphology, flowering, transpiration, photosynthesis and increased rates of proteolysis (Booij-James et al. 2000; Palma et al. 2002). Exposure to high UV-C radiation inhibited the growth, decreased photosynthetic pigment content of shoot and altered the shoot nutritional quality (Liu and Yang 2012). Also, UV-C radiation supplied caused visible injury on leaves, decreased stem length, leaf area and plant height (Kobashigawa et al. 2011). UV radiation increased the production of reactive oxygen species (H_2O_2, O_2^-, OH^-). These oxygen species are extremely reactive and have a cytotoxic nature (Rastogi et al. 2014). Plants have evolved protective mechanisms to keep these deleterious reactions to a minimum *via* enzymatic and non-enzymatic antioxidative defence.

Many reports indicated that the using of natural antioxidant supplements play important roles against abiotic stresses (e. g. high light, UV radiation, temperature extremes, heavy metals, air pollutants, drought stress, salt stress, mechanical/ physical stress). Among the sources of antioxidants are vegetables, legumes, fruits and fruit juices. Spinach (*Spinacia oleracea* L.) is an important dietary vegetable, it belongs to the family Chenopodiaceae. It is known that spinach possesses a wide range of metabolites with biological activity. Several investigators highlighted the important role of spinach extract in micronutrient supply, providing vitamins A, B2, C and K and many other functional elements such as magnesium; manganese; folic acid; potassium and iron (Chatterjee et al. 2010). It is also a good source of chlorophyll, which aids in digestion. Also, spinach extract is a well-known source of the bioflavonoid quercetin and many other flavonoids, carotenoids and polyphenols that protect plant cells against gamma radiation due to their biological and antioxidant activities in addition to many other benefits (Lomnitski et al. 2003; Otari et al. 2010). Moreover, spinach contains a large amount of dietary fibre, vitamins B6, E and omega-3 fatty acids, which are important for the maintenance, improvement and regulation of tissues (Maeda et al. 2010). Bhatia and Jain (2004) pointed to the significant protective effect of a methanolic extract of *Spinacia oleracea* against oxidative stress caused by radiation. Recently, Lamhamdi et al. (2013) pointed to the beneficial effects of spinach extract on improvement wheat plant tolerance against the deleterious effects of lead stress.

Fenugreek (*Trigonella foenum-graecum* L.) belongs to the family Fabaceae, and is a widely known medicinal semi-arid crop, cultivated worldwide. Its benefits had been described in the oldest maintained medicinal document (Ebers Papyrus). It is considered as one of the oldest medicinal plants with medicinal and nutritional values. Many studies have shown that fenugreek seeds contain a large amount of phospholipids, fiber, glycolipids, oleic acid, linoleic acid, linolenic acid, choline, nicotinic acid, vitamins and many other functional elements (Montgomery 2009; Chatterjee et al. 2010; Sadak 2016). In this study, we used spinach extract as a natural antioxidant supplement to examine its antioxidant defence role in alleviating the harmful effects of UV-C radiation in fenugreek plants.

Table 1 Some phytochemicals compounds detected in *Spinacia oleracea* leaves

Class	Compound
Carotenoids	*cis*-Lutein, *trans*-lutein
	cis-β Carotene, *trans*-β carotene
Flavonoids	Glucurinated flavonoid 6-(3,4-dihydroxyphenyl)-9-hydroxy-7-methoxy dioxolo[4,5-g]chromen-8-one 4′-b-glucuronide
	5,3′,4′-Trihydroxy-3-methoxy-6:7-methylenedioxy flavone-4′-glucuronide
	Spinacetine and patuletine (and their glycosides)
Phytoecdysteroids	20-Hydroxyecdysone
Organic acids	*p*-coumaric acid, *o*-Coumaric acid
	Ascorbic acid, ferulic acid

Materials and Methods

Plant Material

The leaves of spinach (*Spinacia oleracea* Linn.) plants were collected during winter seasons from open areas of El-Badrashin district (Giza, Egypt). The plant was identified by the department of botany, college of Science/ university of Ain Shams.

Preparation of Methanolic Extract of *Spinacia Oleracea*

The method described by Lamhamdi et al. (2013) was adopted for the preparation of the methanolic extract of *Spinacia oleracea* leaves. Briefly, 200 g of fresh spinach leaves collected and separated from the stem by sharp knife then, the leaves and soft stalks were chopped into small pieces and kept under an electric fan at room temperature then these were dried fully. The dried leaves were then pulverized using a blender. 20 g of spinach powder was weighed and extracted twice with 200 ml methanol using an ultrasonic bath for 30 min, centrifuged and the supernatant was stored at 4 °C until use. For testing, the extract was evaporated, 2 g of the residue was weighted and redissolved in 100 ml methanol, thus giving a stock solution with a concentration (w/v) of 20,000 ppm. Main phytochemicals of spinach leave extract are presented in Table 1 which have been analyzed by Bunea et al. (2008), Gorelick-Feldman (2008), Perry et al. (2009) and Lamhamdi et al. (2013).

Fenugreek Germination and Seedling Treatment

Preliminary germination experiment was conducted to identify the suitable concentrations of spinach extract (SE) which caused higher germination percentage of fenugreek seedlings. Two concentrations of spinach extract were chosen from the results of the preliminary experiment at 25 and 50 ppm of SE. The pretreatment solutions were freshly prepared by diluting the stock methanolic extract in distilled water and adjusting pH to 5.5 with HNO_3. Seeds of fenugreek (*Trigonella foenum-graecum* L.) were obtained from the Crop Institute, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt. Prior to germination, seeds were surface sterilized with 5% (v/v) sodium hypochlorite for 10 min and then rinsed several times with distilled water. Fifteen seeds were placed in each Petri dish (15 cm) and covered with two sheets of filter paper, then incubated at 25 ± 1 °C and divided into two groups. The 1st group (5 dishes) was moistened with 10 ml of distilled water to serve as the control (non-irradiated and non-SE treated seedlings). The 2nd group was divided into three sets (9 Petri dishes for each set), after 7 days from germination, seedlings of the 1st, 2nd and 3rd sets were transferred to other three sets of small open plastic pots and immediately irradiated with UV-C inside a specific closed chamber at three different exposure periods 30, 60 and 120 min respectively, by fluorescent UV-C tube (6 W) with intensity of 0.15 mw/cm^2—254 nm then returned to the incubator at 25 ± 1 °C. The distance between plantlets and UV-C lamp was 30 cm. At 14 days of germination, the 1st set was divided into three sub-sets. The seedlings of the 1st sub-set were allowed to complete their germination until the end of the experiment and moistened with 10 ml of distilled water (30 min-UVC treatment). The 2nd sub-set was moistened with 10 ml of 25 ppm of SE (30 min-UVC + 25 ppm SE treatment). The 3rd sub-set was moistened with 10 ml of 50 ppm of SE (30 min-UVC + 50 ppm SE treatment). The 2nd and the 3rd sets were subdivided and treated in a similar manner as that of the first set. Treatments with SE were carried out two times, with 1-week interval for both concentrations (at 14th & 21st days).

All samples were collected after 28 days from sowing to assess certain morphological characteristics (shoot and root lengths, number and roots and leaves, and fresh and dry weights of shoot and root) in addition to some physiological and biochemical criteria. Photosynthetic pigments (chlorophyll a,_b and carotenoids) were estimated following the procedure given by Vernon and Seely (1966). Total soluble protein was done by the method of Lowry et al. (1951). The concentration of total protein is exhibited by a color change of the sample solution in proportion to protein concentration, which measured using colorimetric techniques at 750 nm. Total free amino acids were estimated by using the method of Moore and Stein (1954). The content of total phenolic (TPC) was determined in accordance with Shahidi and Naczk (1995). Total flavonoids were estimated by the method of Bushra et al. (2009). Ascorbic acid (vitamin C)

Table 2 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on the growth criteria of fenugreek seedlings during the germination

Treatments	Shoot length (cm)	Root length (cm)	No. of roots/ plant	No. of leaves/ plant	Area of leaves (cm^2)	Fresh weight (mg)		Dry weight (mg)	
						Shoot	Root	Shoot	Root
Control	5.81^{dc}	6.71^{de}	5.01^{c}	2.72^{cd}	2.41^{c}	132^{b}	$26^{b–e}$	9.00^{de}	2.40^{ab}
30 min UV-C	8.92^{ab}	8.85^{bc}	8.80^{b}	6.80^{a}	4.20^{ab}	220^{a}	70^{a}	19.61^{c}	4.61^{ab}
60 min UV-C	4.00^{ab}	4.70^{fe}	3.00^{e}	1.18^{ab}	1.00^{de}	74^{b}	$51^{c–e}$	8.40^{ef}	1.64^{ab}
120 min UV-C	3.93^{fe}	3.65^{fg}	2.51^{d}	1.00^{ab}	0.95^{e}	52^{a}	23^{de}	6.30^{g}	1.41^{b}
30 min UV-C + 25 ppm SE	11.44^{a}	14.32^{a}	13.80^{a}	8.20^{a}	4.81^{a}	260^{a}	72^{a}	21.42^{a}	7.33^{a}
60 min UV-C + 25 ppm SE	10.82^{ab}	13.48^{ab}	10.46^{b}	7.60^{ab}	4.33^{ab}	240^{a}	70^{b}	19.35^{b}	6.80^{ab}
120 min UV-C + 25 ppm SE	10.21^{ab}	11.2^{c}	9.89^{e}	6.50^{ab}	4.10^{de}	230^{a}	$61^{a–d}$	17.85^{c}	6.30^{ab}
30 min UV-C + 50 ppm SE	4.50^{be}	5.12^{d}	4.00^{e}	2.00^{ab}	1.26^{e}	87^{ab}	$20^{a–e}$	8.10^{d}	2.00^{ab}
60 min UV-C + 50 ppm SE	4.31^{fe}	4.20^{fg}	3.00^{d}	1.50^{bcd}	1.13^{de}	78^{b}	$18^{c–e}$	7.60^{f}	1.70^{ab}
120 min UV-C + 50 ppm SE	4.00^{f}	3.72^{g}	2.80^{d}	1.12^{e}	1.08^{e}	55^{a}	16^{e}	6.80^{g}	1.50^{b}

a-g Data presented as means of three replicates. In a column, means followed by different letters indicate a significant difference at $P \leq 0.05$ according to Duncan's multiple range test

was determined as described by MuKherjee and Choudhuri (1983). The content of α-tocopherol was measured following Philip et al. (1954). Peroxides (POX) activity was assayed using the method of Bergmeyer (1974) using Pyrogallol as the substrate. Guaiacol peroxidase (GPX) activity was measured by the method of Zhang et al. (1995). Polyphenol oxidase (PPO) was assayed according to the method described by Kar and Mishra (1976) with some modification. CAT activity was assayed according to the method of Chen et al. (2000). One unit of enzyme is the amount required for the transformation of 1 µmol of substrate per minute. Electrophoretic protein profile of fenugreek leaves was analyzed according to SDS-PAGE technique (Laememli 1970) which relates polypeptide maps, molecular protein markers, percentage of band intensity using gel protein analyzer.

Statistical Analysis

The experimental design and the laboratory tests was a randomized complete block with three replicates. The data were analysed by using analysis of variance (ANOVA) at $P = 0.05$ level and means were separated with Duncan's Multiple Range Test (DMRT). Multiple comparisons between treatments and control were done.

Results

Changes in Growth Parameters

Data presented in Table 2 revealed that exposure of fenugreek seedlings to UV-C irradiation for 30 min caused substantial increase in growth parameters over the control by 53.5%, 31.9%, 75.6%, 150%, 74.2%, 83.5% and 112% for shoot length, root length, number of roots, number of leaves, leaf area, fresh weight and dry weight of seedlings, respectively. However, exposure of seedlings to longer periods of UV-C irradiation (60 min & 120 min) gradually inhibited seedling growth. 120 min of UV-C exposure resulted in greater growth inhibition, decreasing shoot length, root length, number of roots, number of leaves, leaf area, fresh and dry weight of seedlings by 32.3%, 45.6%, 49.9%, 63.2%, 60.5%, 52.5% and 32.4%, respectively, below that of the control. On the other hand, treating of the irradiated seedlings with low concentration of spinach leave extract (25 ppm SE) improves the growth of 30 min UV-C irradiated seedlings and alleviates the harmful effect of prolonged exposure period of UV-C stress (60 & 120 min). In this respect, growth criteria of fenugreek seedlings showed significant increase at all exposure periods by using 25 ppm SE. Whereas, applying of higher concentration of SE (50 ppm) caused a reverse pattern of effect and resulted in substantial growth inhibition particularly at 120 min UV-C irradiation as compared with control. These results indicated that the lower concentration (25 ppm) of the spinach leave extract has a definitive role in improving the growth of fenugreek under UV-C stress.

Changes in Photosynthetic Pigments

The obtained results in Table 3 revealed that after 30 min of UV-C irradiation the values of total chlorophylls (a + b), carotenoids and total pigment contents increased by 56.5%, 55% and 56% versus the control. Whereas, chlorophyll a and total pigments contents were decreased significantly with increasing exposure periods of UV-C irradiation (120 min) as compared with control. On the other hand, treatment of the irradiated seedlings with a lower concentration of SE significantly increased the contents of photosynthetic pigments at all periods of UV exposure as compared

Table 3 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on the photosynthetic pigments of leaves of fenugreek seedlings during germination

Treatments	Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Total pigments (mg g^{-1} FW)
Control	3.75^{de}	2.80^{de}	$2.78^{b–e}$	9.33^{de}
30 min UV-C	5.25^{c}	5.00^{abc}	4.31^{abc}	14.56^{c}
60 min UV-C	3.00^{e}	$1.74^{b–e}$	$1.87^{b–e}$	6.61^{f}
120 min UV-C	2.43^{f}	1.36^{de}	1.34^{de}	5.13^{g}
30 min UV-C + 25 ppm SE	6.62^{a}	6.41^{a}	6.31^{a}	19.34^{a}
60 min UV-C + 25 ppm SE	6.84^{b}	5.83^{ab}	5.71^{ab}	18.38^{b}
120 min UV-C + 25 ppm SE	5.32^{c}	$5.10^{a–d}$	$4.61^{a–d}$	15.03^{c}
30 min UV-C + 50 ppm SE	3.00^{cd}	$3.00^{a–e}$	$1.82^{a–e}$	7.82^{d}
60 min UV-C + 50 ppm SE	3.60^{e}	2.00^{de}	1.75^{cde}	7.35^{f}
120 min UV-C + 50 ppm SE	2.76^{f}	1.89^{e}	1.46^{e}	6.11^{g}

a–g Data presented as means of three replicates. In a column, means followed by different letters indicate a significant difference at $P \leq 0.05$ according to Duncan's multiple range test

with control. While, application of the higher concentration (50 ppm SE) to 60 and 120 min irradiated seedlings caused a significant reduction in total pigments content, the longest period (120 min) was more deleterious and decreased total pigments content by 34.5% below the control.

Total Soluble Protein Content

Leaf protein content was estimated in fenugreek seedlings after different periods of UV-C irradiation (Fig. 1). Decrease in the content of protein is a common phenomenon in UV stress due to the degradation of amino acids of proteins which absorb UV. However, in this study, we observed a significant increase in protein content of UV-C irradiated seedlings at 30 min. Applying of 25 ppm SE caused significant increase in protein content at 30, 60 and 120 min UV-C by 169%, 126% and 70%, respectively, above the control. The obtained data showed stimulatory effects of spinach extract treatment at 25 ppm on protein content of irradiated seedlings compared with both the control seedlings and the seedlings treated with 50 ppm SE.

Free Amino Acids Content

The obtained data presented in Fig. 2 showed the effect of UV-C either alone or accompanied by spinach extract treatments on free amino acids content of the leaves of fenugreek seedlings. Data showed that exposure of the seedlings to low period of UV-C irradiation (30 min) increased total free amino acids by 21.2% above the control. However, increasing period of UV-C exposure to 60 and 120 min caused significant decrease in free amino acids content by 22.1% and 36.8%, respectively, compared with control. Applying of lower concentration of SE (25 ppm) improved the content of free amino acids at all UV-C exposures used in the study. The maximum induction in free amino acids content was recorded in the seedlings exposed to 30 min and 25 ppm SE, since the content increased by 36.4% above that of the control. On the other hand, high concentration of SE (50 ppm) caused significant reduction in the total free amino acids content at 60 and 120 min as compared with control.

Total Phenol and Total Flavonoids Contents

Data in Table 4 show the variation in antioxidant substances expressed as total phenol and flavonoids contents of the fenugreek seedlings exposed to UV-C irradiation and treated with spinach extract during growth. The obtained data revealed that total phenol and total flavonoids contents were significantly increased at 30 min UV-C irradiation by 15.6% and 24.2% respectively, above that of the control. But, exposure of the seedlings to 60 and 120 min UV-C decline the contents of total phenol and total flavonoid as compared with control. Meanwhile, treatment of the seedlings with 25 ppm SE significantly increased total phenol and total flavonoid contents at all periods of UV-C exposures compared with the control. In contrast, using of 50 ppm SE caused significant decrease in phenol and flavonoid contents at 60 and 120 min. The magnitude of reduction was much more pronounced at 120 min UV-C irradiation.

Changes in Ascorbic Acid and α-Tocopherol Contents

Exposure of fenugreek seedlings to UV-C irradiation for 30 and 60 min caused an obvious increase in ascorbic acid and α-tocopherol (vitamins C & E) contents followed by a significant reduction with increasing exposure period to 120 min as compared with control (Table 4). In addition, treatment of the seedlings with 25 ppm SE resulted in a significant increase in ascorbic acid and α-tocopherol contents at 30, 60 and 120 min of UV-C irradiation. On the other

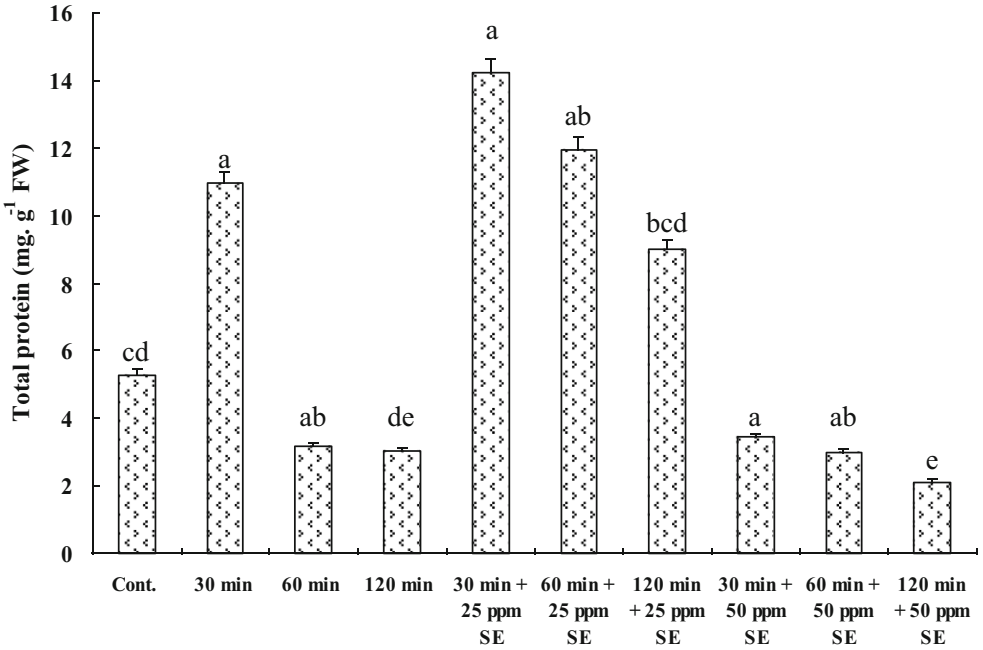

Fig. 1 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on total protein content of leaves of fenugreek seedlings during the germination. Error bars represent the SE ($n = 3$)

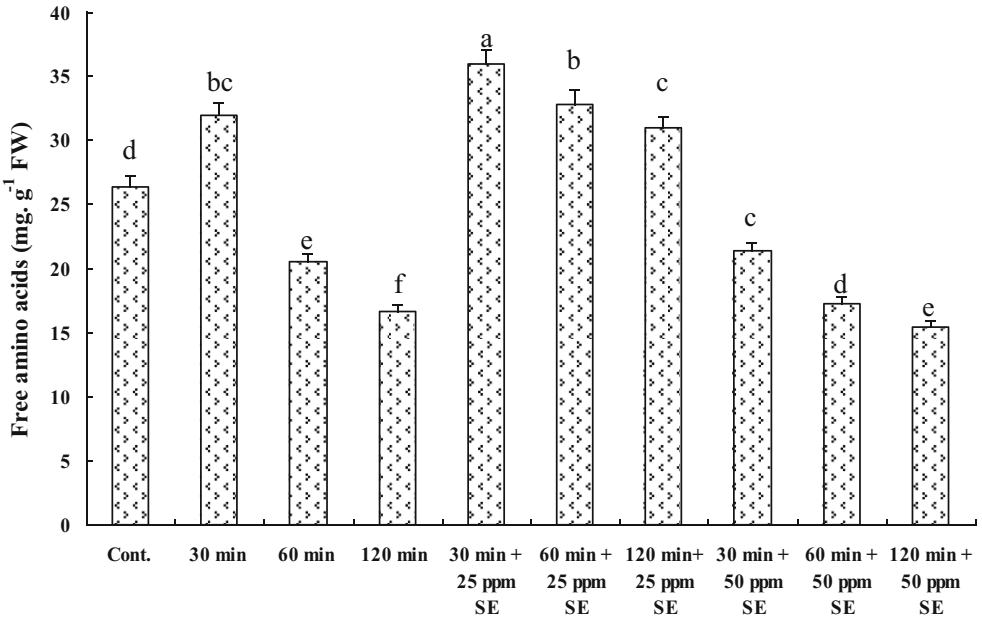

Fig. 2 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on free amino acids content of leaves of fenugreek seedlings during the germination. Error bars represent the SE ($n = 3$)

hand, application of 50 ppm SE caused significant reduction in ascorbic acid and α-tocopherol contents as compared with control.

Antioxidant Enzymes

Antioxidant enzymes activities of fenugreek seedling were also affected by UV irradiation and SE treatments (Table 5). Peroxidase (POX), Guaiacol peroxidase (GPX) and polyphenol oxidase (PPO) activities were significantly elevated by 25 ppm SE at all exposure periods of UV-C irradiation as compared with the control. On the other hand, the activities of POX, GPX and PPO were significantly decreased at 30, 60 and 120 min of UV-C irradiation accompanied by 50 ppm SE. Catalase (CAT) activity was almost unaffected by SE treatments. Its activity was not changed significantly in both SE treatments and corresponding UV-C irradiations. The lower SE concentration had the same effect on CAT activity as the higher one.

Table 4 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on non-enzymatic antioxidants of leaves of fenugreek seedlings during the germination

Treatments	Total phenol (mg catechol g^{-1} FW)	Total flavonoid (mg quercetin g^{-1} FW)	Ascorbic acid (μg g^{-1} FW)	α-tocopherol (μg g^{-1} FW)
Control	109^{e}	8.09^{cde}	5.34^{de}	45.65^{c}
30 min UV-C	126^{b}	10.05^{b}	9.53^{ab}	54.59^{a}
60 min UV-C	92^{c}	5.10^{ab}	4.75^{abc}	37.94^{b}
120 min UV-C	84^{g}	4.83^{a}	4.15^{de}	34.57^{d}
30 min UV-C + 25 ppm SE	138^{a}	13.48^{a}	10.61^{a}	56.19^{a}
60 min UV-C + 25 ppm SE	123^{b}	11.91^{a}	9.11^{ab}	54.99^{a}
120 min UV-C + 25 ppm SE	117^{cd}	9.93^{b}	7.52^{bc}	50.92^{b}
30 min UV-C + 50 ppm SE	102^{d}	7.86^{bcd}	4.37^{c}	41.43^{b}
60 min UV-C + 50 ppm SE	98^{e}	4.70^{a}	3.70^{ab}	36.84^{d}
120 min UV-C + 50 ppm SE	89^{f}	3.20^{b}	3.10^{b}	35.87^{e}

a–g Data presented as means of three replicates. In a column, means followed by different letters indicate a significant difference at $P \leq 0.05$ according to Duncan's multiple range test

Table 5 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on enzymatic antioxidants of leaves of fenugreek seedlings during the germination

Treatments	Peroxidase (POX) (unit. min^{-1} g^{-1} FW)	Guaiacol peroxidase (GPX) (unit. min^{-1} g^{-1} FW)	Polyphenol oxidase (PPO) (unit. min^{-1} g^{-1} FW)	Catalase (CAT) (unit. min^{-1} g^{-1} FW)
Control	60.0^{cd}	65.52^{cd}	19.38^{ef}	215^{ab}
30 min UV-C	77.34^{ab}	71.88^{ab}	27.51^{bc}	222^{ab}
60 min UV-C	$53.8^{a–c}$	53.00^{ab}	16.8^{d}	211^{ab}
120 min UV-C	51.42^{cd}	44.00^{ef}	13.40^{ef}	210^{b}
30 min UV-C + 25 ppm SE	82.12^{a}	76.68^{a}	34.59^{a}	224^{a}
60 min UV-C + 25 ppm SE	79.10^{a}	73.68^{ab}	29.16^{b}	219^{ab}
120 min UV-C + 25 ppm SE	75.22^{ab}	69.72^{ab}	26.28^{c}	211^{b}
30 min UV-C + 50 ppm SE	46.71^{a}	53.90^{e}	14.10^{a}	216^{ab}
60 min UV-C + 50 ppm SE	42.30^{b}	41.00^{ab}	13.51^{ab}	214^{ab}
120 min UV-C + 50 ppm SE	39.15^{e}	33.80^{e}	11.62^{c}	209^{b}

a–f Data presented as means of three replicates. In a column, means followed by different letters indicate a significant difference at $P \leq 0.05$ according to Duncan's multiple range test

Protein Electrophoratic Pattern

The results of SDS-PAGE electrophoretic patterns of protein extracted from the leaves of fenugreek seedlings exposed to different doses of UV-C radiation either alone or accompanied by SE treatments are tabulated in Table 6 and illustrated in Fig. 3. Seven protein bands with molecular weights ranging between 74.31 and 4.33 kDa were separated in fenugreek leaves under control conditions. Exposure of the seedlings to UV-C for 30 min caused the appearance of the same number of total protein bands which was observed in the control (7 bands). In addition, exposure of the seedlings to 60 and 120 min increased the total number of protein bands to 9 bands as compared to the control. Therefore, radiation stress at 60 and 120 min induced synthesis of a new set of protein bands (2 bands) with high molecular weight at 94.57 and 80.56 kDa in case of 60 min and at 105.1 and 80.56 kDa in case of 120 min, respectively as compared with control. In addition, two new protein bands (105.1 & 49.89 kDa) were *de novo* synthesized in fenugreek leaves only at 120 min as compared with both the control and other periods of radiation. The results also showed the appearance of one inducible protein band at molecular weight 7.62 kDa at all exposure periods of UV-C irradiation. On the other hand, protein bands at molecular weight 74.31 and 42.14 KDa disappeared in plants exposed to a higher exposure period of radiation (60 and 120 min) as compared with control. These results indicated that the leaves of fenugreek seedlings irradiated with UV-C for different periods characterized by the appearance of certain new bands and the disappearance of other ones as compared with that of the non-irradiated seedlings. Application of SE extract to the irradiated seedlings induced a considerable variation in the protein patterns of the produced leaves. Table 6 showed that the total number of protein bands at all UV-C exposure periods accompanied by SE treatments was

Table 6 Effect of different exposure periods of UV-C irradiation and spinach extract (SE) treatments on the protein patterns separated by SDS-PAGE of leaves of fenugreek seedlings during the germination

Molecular weight (kDa)	Control	UV-C (30 min)	UV-C (60 min)	UV-C (120 min)	30 min + 25 ppm SE	60 min + 25 ppm SE	120 min + 25 ppm SE	30 min + 25 ppm SE	60 min + 25 ppm SE	120 min + 25 ppm SE
105.1	–	–	–	+	–	–	+	+	+	–
94.57	–	–	+	–	–	–	–	–	–	+
80.56	–	–	+	+	+	–	+	+	–	–
74.31	+	+	–	–	–	+	–	–	+	+
64.44	–	–	+	+	+	–	+	+	–	–
61.93	+	+	+	–	–	+	–	+	+	+
49.89	–	–	–	+	–	+	+	+	+	–
42.14	+	+	–	–	–	–	–	–	–	+
31.46	+	+	+	+	+	+	+	+	+	+
18.84	+	+	+	+	+	+	+	+	+	+
11.58	+	+	+	+	+	+	+	+	+	–
7.62	–	+	+	+	+	+	–	+	–	+
4.33	+	–	+	+	+	+	+	–	+	–
1.73	–	–	–	–	–	–	–	–	–	+
Total No. of bands	*7*	*7*	*9*	*9*	*7*	*8*	*8*	*9*	*8*	*8*
No. of new bands		*1*	*4*	*5*	*3*	*2*	*4*	*5*	*2*	*3*

increased as compared with control except the treatment of 30 min UV-C plus 25 ppm SE which have the same number of total protein band as that of control. In general, both concentrations of SE induced synthesis of a new set of protein bands (six bands) at molecular weights 105.1, 94.57, 80.56, 64.44, 49.89 and 7.62 kDa as compared with control. One inducible protein band which has a molecular weight of 1.73 kDa was newly appeared as a result of exposing the seedlings to 120 min and 50 ppm SE which can be considered as positive marker for UV-C stress. In addition, the same treatment characterized by absence of a protein band at molecular weight 11.58 kDa (negative marker) as compared with the control and all other treatments. Two monomorphic protein bands at molecular weight 31.46 and 18.84 kDa were recorded in the control and in all treatments.

Discussion

The activity of aqueous or alcoholic spinach extracts has been previously described, mainly for their antioxidant (Bergman et al. 2001) and anticancer (Maeda et al. 2010) properties due to the presence of phytochemicals like flavonoids, phenolic acids and pigments like carotenoids, beta carotene, chlorophyll and lutein (Bergman et al. 2001). Along with these chemicals, there are various minerals present in the spinach extract including Mg, Mn, P, Ca, Fe, Zn and Cu in addition to vitamins (A, E, C, K and also folic acid, oxalic acid) which show synergistic effects (Chatterjee et al. 2010). In addition, a powerful natural antioxidant mixture, called NAO, has been isolated from the leaves of spinach (Bergman et al. 2001). This mixture has an antioxidant activity comparable or even superior to that of other antioxidants known. These compounds are scavengers against free radicals and help in decreasing the harmful effects of different environmental factors. These data promoted investigation into whether such protective effect of spinach extract could also be active on the stressed plants.

Exposure of fenugreek seedlings to UV-C radiation induces a lot of metabolic disturbances, which result in a dose-dependent inhibition in the growth parameters of the produced seedlings. In the present study it was observed that exposure of fenugreek seedlings to UV-C at 30 min increased growth parameters. In contrast, longer exposure periods to UV-C caused a reverse pattern of change particularly at 120 min. In this respect, Flint et al. (2003) and Rathore et al. (2003) indicated that UV rays results damage in plants and produce alterations in growth, development and morphology. While Neelamegam and Sutha (2015) reported that groundnut seedlings showed an increment in their growth parameters when the seeds of groundnut were treated with UV-C for 30 min period as observed in the present study. Application of low concentration of SE (25 ppm) improved all growth parameters at 30 min UV-C irradiation and increased growth criteria at 60 and 120 UV-C irradiation. These results are in agreement with that obtained by Lamhamdi et al. (2013) who reported

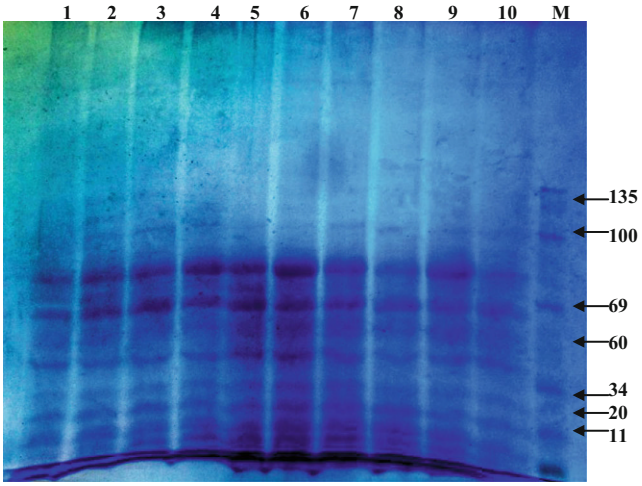

Fig. 3 Electrophoretic banding profiles of protein extracted from the leaves of fenugreek seedlings in response to different exposure periods of UV-C irradiation and spinach extract (SE) treatments. *M* Marker protein, Lane 1: Control. Lane 2: UV-C (30 min). Lane 3: UV-C (60 min). Lane 4: UV-C (120 min). Lane 5: 30 min + 25 ppm SE. Lane 6: 60 min + 25 ppm SE. Lane 7: 120 min + 25 ppm SE. Lane 8: 30 min + 50 ppm SE. Lane 9: 60 min + 50 ppm SE. Lane 10: 120 min + 50 ppm SE

that the extract of spinach leaves has a promoting effect on the length and the biomass of shoots and roots of wheat seedling under lead stress. The increasing in roots length of the fenugreek seedlings at all exposure periods of UV-C plus 25 ppm SE treatment may be due to presence of carotenoids in SE. Carotenoids play an essential role as a stimulating factor in gibberellin biosynthesis which controls the growth of root by regulating cell elongation. Besides, the presence of vitamins as coenzymes play important roles in plant growth, various physiological events and antioxidant defense system (Athar et al. 2009). In contrast, application of 50 ppm SE decreased fenugreek growth, particularly at 120 min UV-C irradiation (Table 2). We will thereafter focus on the effects of the treatment with 25 ppm SE in the following discussion.

The experimental results showed that 30 min of UV-C radiation increased the contents of chlorophyll a and chlorophyll b, leading to an increase in total pigments content. This induction in pigment contents may be due to the stimulating effect of 30 min UV-C on growth criteria particularly leaf area. On the other hand, the reduction in chlorophyll content under UV-C stress at 60 and 120 min has a negative effect on total pigments content and plant photosynthetic efficiency since photosynthesis is dependent on chlorophyll content. These results are in harmony with those obtained by Mahdavian et al. (2008) who reported that the contents of chlorophyll a, b and carotenoids of pepper leaves were reduced significantly in plants exposed to UV-C radiation. The promoting effect of 25 ppm SE on chlorophyll-a, carotenoids and total pigments contents at all UV-C exposure periods may be due to increase of chlorophyll synthesis, since SE have an important role in micronutrient supply, providing vitamins A, B2, C and K; manganese; magnesium; iron; folic acid and potassium which improve mineral uptake and enhancing the content of leaf chlorophyll of fenugreek seedlings treated with spinach extract. Spinach is a good source of the bioflavonoid quercetin with many other flavonoids which exhibits protection against gamma radiation (Otari et al. 2010). Carotenoid content increased in UV-C irradiated seedlings treated with 25 ppm SE at 30 and 60 min. Carotenoids are essential pigments which perform a photoprotective function where they aid in the photosynthetic process of converting it into metabolic energy and also function as antioxidants by neutralizing free radicals that could harm the plant.

Total protein content of fenugreek seedlings also increased at 30 min UV-C irradiation (Fig. 1). It is reported that, increased levels of protein can prevent UV-C induced damage to plant tissues and plant with increased levels of protein show decreased sensitivity to damage by UV irradiation (Nasibi and Kalantari 2005). This increment may be related to defense enzymes and proteins which are probably synthesized during stress. Application of 25 ppm of SE significantly increased protein content of the irradiated seedlings at 30 and 60 min as compared with control (Fig. 1). The increase in protein content is possibly due to the induction of stress proteins, which may include various antioxidant enzymes synthesized during stress. Moreover, this increase in total protein content of 25 ppm irradiated seedlings might be a concomitant with the enhanced growth rate of the treated seedlings as a result of increased photosynthetic performance, which would lead to a predicted increase in the nitrogen pool. The enhancement in the total free amino acids content by using 25 ppm SE which was observed in this study supports this view through their incorporation into protein. Al-Dosari (2010) reported that spinach extract was able to restore the levels of non-enzymatic antioxidant biomarkers, such as proline and other amino acids, and non-protein sulfhydryl in the tissue previously stressed with carbon tetrachloride. The accumulation of proteins provides protection to the plant during radiation stress (Ramya and Balakrishnan 2013). The increment in free amino acids at 30 min UV-C treatment could be due the role of these compounds in the protection from oxidative stress, since plant tissues possess the ability to activate a wide variety of individual amino acids under stress conditions (Martino et al. 2003). On the other hand, the reduction in total free amino acids content at 60 and 120 min of UV-C could be considered as an indication of the damage to fenugreek seedlings due to the poor metabolic adaptation to severe stress caused by UV radiation. Application of 25 ppm SE caused a marked increase in total free amino acid content of UV-C irradiated seedlings. These results

may be attributed to the spinach extract content which might induce tolerance to UV stress. It was shown that spinach extract contains secondary metabolites such as carotenoids or phytosterols which are considered as a means for plants to interact with the environment and protect plants against both abiotic and biotic stress since phytosterols are integral components of the plant cell membranes and are responsible for its fluidity and permeability (Posé et al. 2009; Abd El-hamid et al. 2016).

The present study pointed out that flavonoids and other UV-absorbing compounds, including polyphenols, ascorbic acid and α-tocopherol which have many antioxidative properties, are synthesized in large amounts in response to UV at 30 min. Therefore, mild levels of UV-C radiation may be an alternative to increase the content of antioxidants and other phytochemicals substances in fenugreek seedlings. These compounds play an important role against UV damage in higher plants through preventing the penetration of destructive bands of UV light to the most sensitive tissue (Nasibi and Kalantari 2005). Our results are consistent with those of many previous studies showing the accumulation of flavonoid and phenolic compounds in plants in response to UV irradiation (González-Aguilar et al. 2007). In this respect, Rahimzadeh et al. (2011) found that flavonoids were induced and accumulated in savory plant tissues in response to UV-C irradiation. Many researchers highlighted that the consumption of different antioxidants can provide protection against oxidative stress. Spinach extract is a good source of flavonoids, carotenoids and polyphenols which having scavenging properties (Lomnitski et al. 2003; Otari et al. 2010). Phenolic compounds in SE may produce the beneficial effects by scavenging free radicals (Chun et al. 2003). Thus, phenolic compounds may protect cells against the oxidative damage caused by UV-C stress. Moreover, flavonoids appear to be the major contributors to antioxidant capacity in spinach. Ascorbic acid (vitamin C) is a key non-enzymatic antioxidant that serves as a cofactor for enzymes involved in photosynthesis, hormone biosynthesis, and regeneration of antioxidants which protect plant cells against oxidative stress. It has been implicated in control of plant growth and development through its interaction with phytohormones. It plays a pivotal role in consuming the excessive light energy and protecting photosynthesis for plants. Scavenging the ROS is one of the major tasks of ascorbic acid by maintaining ROS at non-damaging levels. It regulates the synthesis of most enzymes responsible for the development of important metabolites and increases the natural antioxidant activity (Sajid et al. 2017). The obtained results revealed that treatment of the irradiated seedlings with SE particularly with the lower concentration (25 ppm) increased ascorbic acid and α-tocopherol contents. Several investigators have pointed out that vitamin E (α-tocopherol) is one of the best quenchers for singlet oxygen, and can act as a chain-breaking antioxidant. Considerable evidence has been accruing of the importance of α-tocopherol and retinol in protecting lipids and other membrane components from damage by reacting with singlet oxygen. Fornaciari et al. (2015) pointed to the ability of spinach extract to counteract with oxidative stress.

In this study, the reduction in peroxidase and guaiacol peroxidase activities which was observed at 60 and 120 min UV-C stress might be due to a decline in enzyme synthesis, or a change in the assembly of enzyme subunits under stress. Applying of 25 ppm SE to irradiated seedlings resulted in higher activities of POX, GPX and PPO. Since the biological function of POX and GPX is the removal of H_2O_2 and other hydroperoxides, so that their higher activities would be beneficial for protection against lipid peroxidation and DNA hydroperoxides in UV-C stressed fenugreek seedlings. Furthermore, increase in POX activity might be the result of increase in *de novo* protein synthesis or the activation of enzymes already present in plant cells to diminish the harmful effects of ROS. Balakrishnan et al. (2005) reported that peroxidase and polyphenol oxidase serve as acclimatisation mechanisms to scavenge the toxic free radicals of oxygen produced under stress conditions. The induction in PPO activity can be considered as an evidence for the vital role of lower concentration of SE in increasing PPO activity. On the other side, experiments on overexpression of antioxidant production do not always result in the enhancement of the antioxidative defense, and hence increased antioxidative capacity does not always correlate positively with the degree of protection (Gout et al. 2001). Here in the present study, CAT activity showed no significant change as a result of UV-C irradiation either alone or accompanied by SE treatments. Similarly, Jain et al. (2004) did not observe an increase in the activity of antioxidant enzymes by UV-radiation in the presence of external supply of antioxidants.

The obtained results from SDS-PAGE analysis showed the presence of new synthesized protein bands with high molecular weights in response to exposure of the seedlings to UV-C stress and SE treatments as compared to control. These new protein bands can be considered as a stress protein in fenugreek seedlings, which were suggested to protect the cell against the adverse effect of UV stress. Similar work has been done by Charles et al. (2009). They also reported the synthesis of 5 new proteins under UV-C stress. The obtained results revealed that UV and/or growth regulators (in SE) altered the protein synthesis patterns due to the presence of several osmoresponsive genes involved in adaptation to UV (Britto et al. 2011). Protein profile of fenugreek seedlings leaves indicated that SE components regulated the expression of UV-stress inducible proteins as well as induced *de novo* synthesis of specific polypeptides

and hence, they may play an important role in UV resistance (Britto et al. 2011).

Conclusion

This study shows that exposure of fenugreek seedlings to mild stress of a UV-C (30 min) has a positive effect on growth parameters and chemical constitutes of the grown seedling. But longer exposure periods of UV-C caused harmful effects on these parameters. Application of lower concentration of spinach extract (25 ppm) as a natural antioxidant supply was more effective in improving seedling growth at 30 min exposure and overcoming the negative effects of UV-C stress at longer exposure periods. So, this work recommended that application of lower concentrations of spinach extract could act as a protective agent to nullify the influence of UV-C stress on fenugreek seedlings with emphasis on the use of this renewable bioresource in agricultural systems.

Conflict of interest A.H.M.A. Mohammed and S.A. Akladious declare that they have no competing interests.

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Abdel Haleem Mohammed Ahmed Mohammed Associate Professor of Plant Physiology Faculty of Education, Biological and Geological Department, Ain Shams University. PhD degree 1998 (Ain Shams University, Faculty of Education, Biological and Geological Department). Title of Thesis "Physiological responses of plant to flooding". Master degree 1991 (Ain Shams University, Faculty of Education, Biological and Geological Department). Title of Thesis "comparative physiological studies on tall and dwarf beans (*Phaseolus vulgaris*)".