Effects of sulfur on total glucosinolate content of *in vitro* regenerated plants of oilseed rape (*Brassica napus* L.)

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Summary: The influence of different concentrations of $MgSO_4$ (basal salt) on total glucosinolate contents in seeds and leaves of hypocotyl and cotyledonary regenerated plants of canola and rapeseed was studied. For callus induction and plant regeneration, explants were cultured on MS medium containing modified two and four folds of the basal salt $MgSO_4$ concentration. Total glucosinolate contents in the seeds and leaves of mature regenerated plants were determined using UV-Vis Spectroscopy. The high concentrations of $MgSO_4$ basal salt increased the glucosinolate contents in the seeds as well in the leaves of the canola and rapeseed regenerated plants.

Introduction

Glucosinolates (GSL) are a class of about 120 naturally occurring thioglucosides that are found in 16 dicotyledonous families of Angiosperms, mainly *Brassicaceae* and *Capparaceae* [1].Glucosinolate is a sulfur-containing compound and majority of cultivated plants containing glucosinolates belong to the cruciferae family. Many of the crucifers that serve as sources of food and condiment belong to the genus *Brassica.* Glucosinolates occurring in the cultivated plants are responsible for the pungent flavors of the condiment horseradish and mustard. They are also responsible for characteristic flavors of turnip, rutabaga, cabbage, and related vegetables. In cruciferous plants, some of these substances have also been associated with endemic goiter hypothyroidism with an enlargement of the thyroid gland [2]. Crucifers often grow in pastures and are consumed by livestock. Rapeseed, derived from varieties of *Brassica napus (B. napus)* and *Brassica campestris (B. campestris*), is one of the major oilseeds of commerce. Glucosinolates occur throughout a plant in the root, stem, leaf, and seed. They are always accompanied by a thioglucosidase enzyme system capable of hydrolyzing the glucosinolates, when wet raw plant material is crushed. During this process glucose and aglucon are always released as products. The aglucon portion can produce a nitrile often with the lose of sulfur or may undergo an intra molecular rearrangement to give an isothiocyanate [3], or thiocyanate. All glucosinolates contain β-D-thioglucose as the sugar moiety. The glucosinolates are anions and usually occur in plants as potassium salts. Although glucosinolates are present throughout the plant, their highest concentration is usually in the seed. However, disease and insect resistances of these plants may be associated with higher glucosinolate content. Glucosinolates may act as attractants to some insects or as repellents to others [4].

All crucifers contain glucosinolates, which upon hydrolysis provide much of the culinary flavor characteristic of these plants. Consumption of cyanogenic foods has been considered as one of the etiological factors in certain instances for the persistence of endemic goiter. *Brassica* family vegetables not only inhibit thyroid production, but they also inhibit cancer growth [5]. It has been shown that some of the nitrile, aglucon products are liver or kidney toxins. Glucosinolates need to be examined as sources of goitrogens as well as toxicants to other body organs [6]. It is known that sulfur, copper, and iron work closely together and that excessive sulfur can reduce iron. Previous study has mentioned that excessive kale consumption will cause anemia in cattle. Generally, anemia is the result of low iron and/or copper. Foods and drugs resulting into anemia also reduce cancer growth, indicating that the *Brassica* vegetables might reduce cancer by inducing anemia [7]. The presence of GSL in the seeds of economically important plant species, such as canola (*Brassica napus* L) is undesirable

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because of the toxicological effects of their breakdown products [8]. GSL occur in the cell vacuoles throughout the vegetative and reproductive tissue in *Brassica* plants but in widely different concentrations [9]. The initial content of the GSL in the seeds and vegetative parts such as leaves of the plant can be influenced and modified by a number of factors such as moisture [10-13], temperature [14], air composition [15], light [16] and also in different growing seasons [17]. The contents of GSL in the *Brassica* plants are also sensitive to the application of chemicals such as fertilizers and pesticides [18-20]. The GSL are mainly consisted of sulfur compounds and these compounds play essential role in the defense mechanism of plant against the infections caused by the pathogens [21-24]. Tissue culture techniques can be used for inducing variation like somaclonal variation [25], micro spore [26-27] and protoplast culture. These techniques have successfully assisted conventional plant breeding and their combination has resulted in significant improvements in the studies of plants. Tissue culture technique involves callus induction from explants, morphogenesis, shoot development and finally root development to regenerate into complete somaclone.

All these steps require a growth medium for developing somaclonal variants successfully. For research studies, the concentrations of some of these chemicals are lowered, enhanced or altogether omitted to see the effect of altered culture conditions on the species [22].

In the present research studies, the concentration of sulfur was changed in the MS medium [23] to observe its effects on the total GSL content in the regenerants derived from canola or rapeseed (*Brassica napus* L) plants.

Objective

1. To study the reduction of GSL contents in seeds of oilseed *Brassica* for utilization as rich source of nutrition for animals
2. To study the increase of glucosinolate content in leaves and stem of oilseed *Brassica* as an insect and pest repellent.
3. To study the effect of different concentrations of sulfur in MS medium on total glucosinolate content.

Materials and methods

The healthy and uniform seeds of three genotypes viz. Excel, PO-3 and RM-159-2 of *Brassica napus* L species were used for the present study. The initial GSL content of each genotype is presented in Table- 1. The sterilized seeds of the genotypes were germinated on half strength hormone-free MS medium of pH 5.8 with 1% sucrose and 0.7 % plant agar.

Table-1 The initial GSL content (µmol/g) in seeds of three genotypes of the *Brassica napus*

Genotype	Quality Status	GSL (µmol/g) in seeds	GSL (µmol/g) in leaves
Excel	Canola*	14.5	16.3
PO-3	Canola	42.6	13.6
RM-159-2	Rapeseed**	70.5	22.4

* Low Erucic Acid
** High Erucic Acid

About 20 seeds were plated on the germination medium in each petridish. This allowed the germinating seeds to grow taller and straighter which made it easier to harvest the cotyledons/ hypocotyls explants. The plated seeds were kept at 25 ^{0}C in the growth chamber. Three types of media i.e. Medium I, Medium II and Medium III were prepared by using MS medium with 3 % sucrose, 4.5 mg/ L benzyl adenine (BA), PH 5.8, and solidified with 0.7 % agar. Medium-II was prepared from MS medium with two times the concentration of Mg SO_4 and Medium-III was prepared from MS Medium with four times the concentration of Mg SO_4. The cotyledons and hypocotyls were cut from the developed seedlings within one week of germination. Twenty healthy and fully unfolded cotyledons (from 10 seedlings) were dropped on the surface of a plate of each Medium. Similar procedure was adopted for the hypocotyls. Callus induction occurred within 2-3 weeks in the cotyledons and hypocotyls explants. Once a good root system was formed, the plantlets were transferred to moist potting soil. The total GSL contents were determined in the leaves and seeds of regenerated plants using UV-Vis Spectrophotometer. The study was conducted in Randomized Complex Block (RCB) Design with Split Plot arrangements using media with different sulfur concentrations as main plots and the genotypes as sub plots with four replications. The data were analyzed with Mstat-C statistical package.

Results and Discussion

Cotyledons regeneration

The canola quality genotype Excel showed about 11.27 % increased GSL content in the seeds of mature plants regenerated through cotyledons explants when the four fold increased concentration of sulfur ($MgSO_4$) was added in the culture medium-III. The effect of high concentrations of $MgSO_4$ with the standard MS medium [23] in case of cotyledon regeneration is presented in Table.2 (Fig.1). The two fold increase in $MgSO_4$ concentration in medium-II accumulated 4.90 % more GSL in the seeds of Excel genotype. The increased $MgSO_4$ concentration also enhanced the GSL content in the leaves of this canola genotype as compared to the control plants with 3.7 % and 6.10 %, respectively.

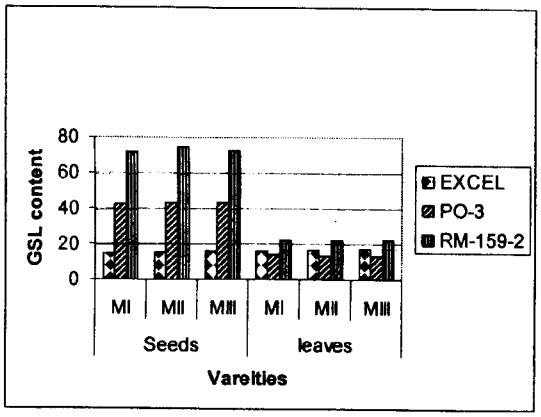

Fig.1 Total GSL content in seeds and leaves of regenerants of cotyledon of different cultivars of Brassica napus

Table- 2. Total GSL (µmol/g) content in seeds and leaves of regenerates of Cotyledon of different cultivars of *Brassica napus*

Genotypes	Seeds			Leaves		
	MI	MII	MIII	MI	MII	MIII
EXCEL	14.2	14.9	15.8	16.2	16.8	17.2
PO-3	41.9	43.1	42.7	13.5	13.1	13.3
RM-159-2	71.5	74.5	72.3	22.3	22.1	22.0

MI= Normal concentration of $MgSO_4$
MII= Two fold high MgSO4
MIII= Four fold high MgSO4

In case of canola genotype with high GSL "PO-3", the medium-II and III increased the GSL content in the mature seeds of the regenerated plants. But the medium-II accumulated more GSL as compared to medium-III (2.86 % and 1.91 %). Both higher concentrations of $MgSO_4$ decreased the GSL content in the leaves of the regenerated plants of PO-3 genotype as compared to control plant (Fig.1 and Table- 2).

The medium-II with two folds high $MgSO_4$ also increased the GSL content up to 4.15 % in the seeds of the cotyledon regenerated plants of the rapeseed (non-canola) genotype "RM-159-2 (Fig.1). The four folds increased $MgSO_4$ medium-III decreased the GSL content (1.48 %) in the leaves of the regenerated plants of rapeseed as observed in case of genotype PO-3.

Hypocotyls regeneration

The GSL content of mature seeds of the hypocotyls regenerated plants clearly increased with the modification in the normal MS media. In case of "00" Excel genotype, 11.41 % increase in the GSL content in the seeds of the regenerants was observed as shown in Table- 3 (Fig.2). The high concentrations of $MgSO_4$ also enhanced the GSL accumulation in the leaves of the canola quality genotype as showcased in the Table- 3.

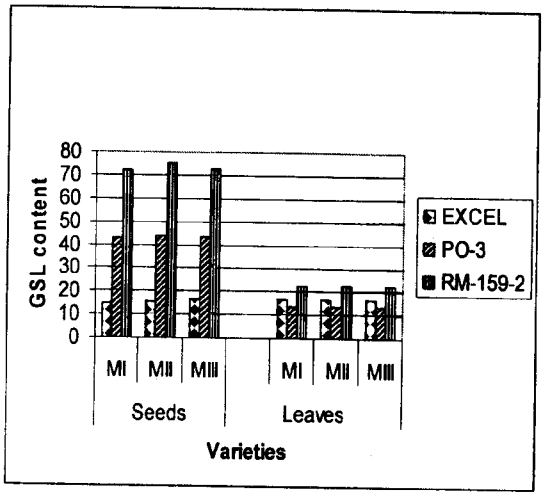

Fig.2 total GSL content seeds and leaves of regenerants of Cotyledon of different cultivars Brassica napus

The Medium-II and Medium-III increased the GSL contents in the seeds of the "0" genotype PO-3. The reduction in the GSL content in the leaves of this genotype was noted with the modified MS media (Table- 3).

Table3. Total GSL content (µmol/g) in seeds and leaves of regenerate of hypocotyls of different cultivars of *Brassica napus*

Genotypes	Seeds			Leaves		
	MI	MII	MIII	MI	MII	MIII
EXCEL	14.9	15.6	16.6	16.5	16.4	16.8
PO-3	42.6	43.8	43.4	13.8	13.7	13.6
RM-159-2	72.3	75.3	73.1	22.6	22.4	22.3

MI= Normal concentration of $MgSO_4$
MII= Two fold high MgSO4
MIII= Four fold high MgSO4

In case of non-canola rapeseed genotype "RM-159-2", Medium-II (two fold $MgSO_4$) increased the GSL content in the seeds of the regenerated plants. The Medium-II only increased the GSL content 3.0 µmol/ g as compared to normal grown plants. The higher concentrations again decreased the GSL content in the leaves of the rapeseed genotype (Table- 3).

In *vitro* studies on GSL content in different species of *Brassica* has been carried out by a number of scientists in order to observe the effects of sulfur levels [9, 21] on the presence and amount of this important compound. Poulsen [24] studied the calluses development and growth in *B.napus* from hypocotyls explants. These explants cultured on MS medium supplemented with 1, 2 and 4 times of basal $MgSO_4$ content. The modified concentrations of $MgSO_4$ enhanced the growth and development of the calluses induction but the GSL content noted with changed concentrations in early regeneration stages.

Microspore derived embryos of *Brassica napus* were used to study the influence of sucrose, jasmonic acid and abscisic acid on total GSL content [28]. The different compositions of these compounds significantly affected the GSL content in the developing embryos. Callus and suspension cultures established from *Nasturtium montanum* and *Cleome chelidonii species* [29]. Large increases in GSL hydrolysis products noted when cultures were supplemented with l-cysteine and l-methionine, and further increases produced in *N. montanum* with l-tryptophan supplementation.

Conclusions

The overall results of the present studies regarding the GSL content in the fresh leaves and mature seeds of the regenerated plants of various genotypes of *Brassica* in both canola and non-canola quality revealed that the enhanced concentrations of sulfur ($MgSO_4$) clearly expressed its effects on the oil quality of the seeds as well as changing the content of the GSL in the leaves of the all under observation genotypes. The media concentration has a very significant role in the practical application of the *in vitro* techniques to improve the genetic architecture of the oilseed *Brassica* species especially in case of quality improvement breeding programs.

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