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Quantitative analysis of Sinigrin in *Brassica Juncea*

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Abstract
It has been reported that *Brassica juncea* (Indian mustard) contains significant amounts of aliphatic glucosinolate, sinigrin. Quantitative analysis of sinigrin was done in thirty genotypes of *Brassica juncea.* All the genotypes were grown in the field and samples of different plant tissues were collected from randomly selected plants for High performance liquid chromatography (HPLC) analysis. Significant variation was found among the genotypes as well as within the plant. Sinigrin was found significantly higher in reproductive part then the other plant parts. Concentration range of sinigrin in stem of thirty genotypes was from 9.4 µmol/g DM (Kranti) to 32.46 µmol/g DM (RH-8701). In leaves it ranged from 14 µmol/g DM (Varuna) to 64 µmol/g DM (B-85 glossy). As compare to vegetative parts in reproductive parts, sinigrin was found higher in concentration. It ranged from 31.02 µmol/g DM (Kranti) to 73.49 µmol/g DM (T-6342) in floral part.

Keywords: Glucosinolate, Genotypes, Quantitative, Sinigrin, HPLC

Introduction
Brassica juncea is an important oilseed crop cultivated worldwide in central and south Asia, Europe and North America, Northern Africa and China, belongs to the *Brassicaceae* family. In India it is commonly known as Indian mustard.
Many beneficial properties have been attributed to cruciferous vegetables [1, 2]. Numerous members of the *Brassicaceae* family are commercialized for animal and human consumption around the world as a rich source of nutrients and also, as healthy products [3]. Consumers not only appreciate vegetables for their nutritional value, but also for their contents in compounds that produce benefits for human health so the active principles of vegetables constitute nowadays the basis of most pharmacological substances. In this sense, it is essential to analyze the possible antimutagenic, anticarcinogenic and anti-aging activity of a botanical nutraceutical substance. In India, *B. juncea* is one of the most economically important crops, cultivated as an oil and leafy vegetable plant it contains phytochemicals such as phenolics, vitamins, minerals and glucosinolates like all other crucifers. Glucosinolates (β-thioglucoside-*N*-hydroxysulphates, GLSs) are naturally occurring thioglucosides that are characteristic of the *Brassicaceae* (including the genus *Brassica*) and related family in the order *Capparales* [4].
Glucosinolates (GSLs) are nitrogen and sulfur rich secondary metabolites grouped into three major groups, namely aliphatic, indolic and aromatic glucosinolates, according to their side-chain structure which represent the precursor amino acids involved in the biosynthesis of the individual compound [5] via independent metabolic pathways, and share a common set of enzymes that is involved in the core structure formation of glucosinolates under genetic control [6]. Upon tissue damage, these compounds are hydrolyzed by β - thioglucoside glucohydrolases called myriosinases into various biologically active compounds like isothiocyanates (ITC) and nitriles. Inherent as defense compounds, protect the plants against a wide range of herbivores, insects and pathogenic invaders. Breakdown products of these compounds also impart aroma and flavour to Brassicales which finds its utilization in culinary purposes from time immemorial. Glucosinolates are found to have detrimental activity against various types of cancer such as breast, lung and colon and regular consumption of crucifers is shown to be effective in the reduction of the risk of cancer [7]. They are also known to possess anti-nutritional properties [8] although produce different effects in humans in function of their chemical structure and concentration in the plant. Both vary considerably depending on the species, stage of development, type of tissue and environmental conditions [3].
There are different types of aliphatic glucosinolates, among them, the final product in 3C glucosinolate pathway, sinigrin, is considered to have great importance in human health as well as in plant defence processes. Degradation product of sinigrin, allyl isothiocyanate has been shown to have bactericidal and antimicrobial activities [9], anti-proliferative activities

against liver [10] and bladder cancer [11]. Sinigrin and its degradation products suppress nitric oxide production in macrophages [12] and reduce plasma triglyceride level [13]. Due to its beneficial role, sinigrin is now even available as a nutrition supplement. In plants it is known to trigger stomatal closure by acting on K+ channels [14], prevent water loss and provide defence against fungal invasions [15] and bacterial pathogens [16].

The concentration of glucosinolate has been found to vary between *Brassica* species as well as between cultivars of the same species (17-19). Different tissues of the same plant also present quantitative and qualitative differences in their glucosinolate content, with other factors such as plant age and environmental growth conditions influencing glucosinolate profile (17, 20).

To maximize the potential benefits it is essential to evaluate glucosinolate levels in *Brassica* species and to investigate the plant tissues that will give the required concentration of glucosinolate.

The aim of the present study was to investigate the glucosinolate (sinigrin) concentrations in different plant tissues of *B. juncea*

Materials and Methods

Plant material

Thirty genotypes of *Brassica juncea* comprising varieties and advanced genotypes were collected from different centres of the All India Coordinated Research Project (AICRP) on rapeseed and mustard, Sewar, Bharatpur (Rajasthan), India. The detail of the genotypes under present experiment is given in table1. The genotypes have important features and were previously characterized morphologically.

Field plot technique

The experiment was conducted at the field of HLRDC, Hisar in Randomized Block Design (RBD) and replicated three times in a plot size of paired rows of 4 m length. The genotypes were grown under recommended agronomic Package of Practices (21) at 30 cm row to row and 10-15 cm plant to plant spacing. Fifteen days after germination, thinning was carried out manually to maintain the optimum plant population. At time of sowing the 40 kg N/ha, 40 kg P_2O_5/ha and 40 kg K_2O/ha was applied and 40 kg N/ha was applied at time of first irrigation (45 days after sowing). Sampling was done when the plants were mature (samples collected 80 days after sowing). All samples were taken on the same day.

Analysis of sinigrin

Analysis of sinigrin was performed as the extraction of glucosinolates, conversion to desulfoglucosinolates and analysis by HPLC according to according to the 'NF EN ISO 9167-1' method as suggested by Haddad and Allaf [22].

200 mg of plant material per sample was used for the sinigrin extraction. Plant samples were freeze-dried prior to extraction. All samples for each variety were analyzed in triplicate in independent experiments.

For extraction, samples into 10 ml tubes were kept into a water bath at 75ºC for 1 minutes 2 ml of boiling methanol (70 percent) was added in tubes, again kept for 1 hr in the water bath and shaken every 20 minutes Then tubes were cooled at room temperature before being centrifuged at 5000 rpm for 3 minutes the supernatant was poured into new tubes. 2 ml of boiling methanol (70 percent) was added to the tubes containing the sediments (oil cakes) the procedure was repeated as before. Finally, the supernatants were adjusted with pure water and homogenized.

The extracts were purified on an ion-exchange column containing 1 ml sephadex DEAE A25 and allowed to run off. Then, 1 ml of Ammonium acetate buffer (2M, pH 4) was added twice and allowed to run off. 75 µl of purified sulfatese (E.C. 3.1.6.1, type H-1) (Sigma) solution were then added and left overnight at room temperature. The desulfoglucosinolate obtained was eluted with 4 ml of water and analyzed by HPLC.

The quantification of sinigrin was done through HPLC analysis using a Shimadzu SPD-20 AT apparatus coupled with a PDA detector was used and the analysis protocol was the following: 20 µl of each sample was run on a NUCLEOSIL C18 column at the flow rate of 1 ml/min, the mobile phase was methanol: phosphate buffer solution pH=7, (95:5); temperature of column was 35°C. Compounds were detected by UV at wavelength of 227 nm. Identification of most of the HPLC peaks was based on a comparison of retention time and UV absorption spectrum as determined on a photo-diode-array detector with those of purified standard. Briefly, we run the standard sinigrin (Sigma-Aldrich) for each independent experiment to calibrate the instrument. The quantification of sinigrin present in the sample was calculated from HPLC peak areas using the sinigrin standard as a reference. Quantities results were given as µmol/g DM tissue (based on the mean of the three extractions for that genotype).

Statistical analysis

Analysis of variance (ANOVA)

Means were separated using the least significant difference (LSD) test at probability of P = 0.05 with the statistical software OPSTAT [23].

Result and Discussion

Peak of standard (sinigrin) was obtained at retention time 2.427 minute shown in figure 1. Stoin *et al*. [24] and Michael [25] also observed the sinigrin retention time at 2.26 minutes in *Brassica nigra* and *Brassica juncea* respectively.

Sinigrin concentration in leaves of thirty genotypes of *Brassica juncea* ranged from 14 µmol/g DM (Varuna) to 64 µmol/g DM (B-85 glossy) as shown in figure 2. PM, T-6342 and B-85 glossy had higher amount of leaf sinigrin, 45.2 µmol/g DM, 46.6 µmol/g DM and 64 µmol/g DM respectively. Concentration of sinigrin in stem ranged from 9.4 µmol/g DM (Kranti) to 32.46 µmol/g DM (RH-8701) shown in figure 3. In flower, sinigrin was found higher in concentration as compare to vegetative parts. It ranged from 31.02 µmol/g DM (Kranti) to 73.49 µmol/g DM (T-6342). In all the thirty genotypes sinigrin content of individual organs varies considerably. The flowers had the highest concentration (figure 4). These organs had over 60 µmol per gram dry weight, at least twice as high as all of the other two organs.

Plants that produce glucosinolates commonly accumulate them in all vegetative and reproductive parts throughout development [26]. Sinigrin, was present at significantly higher levels in flowers than in leaves and stem part. The higher sinigrin content observed in the flowers then the next higher levels followed leaves and stems in all the genotypes. These findings are similar to those found in other glucosinolate-containing species studied by Fahey *et al*. [27]. Brown *et al*. [28] concluded that the organs attached to the bolting stem varied in glucosinolate content. They observed that the

inflorescences had a composition much like that of rosette leaves, but a higher concentration.

Bellostas *et al.* [29] also observed higher glucosinolate content in the reproductive organs compared to the other vegetative parts in four *Brassica* species of the U-triangle, namely *B. nigra* (L.) Koch, *B. carinata* Braun, *B. juncea* (L.) Czern. and *B. rapa* L. Kumar *et al.* [30] found total glucosinolates differed significantly among genotypes and was also significantly influenced by the developmental stages and plant parts. Glucosinolates in leaves were, in general, higher than that of stem (Smallegange *et al.* [31] studied that sinigrin was by far the most abundant compound in all three *Brassica nigra* genotypes with levels at least 44 times higher in leaves and at least 87 times higher in flowers of any of the three genotypes. Gupta *et al.* [32] identified the individual glucosinolates in extracts from the seed and leaf tissues of *B. juncea* lines. Sinigrin component ranged from 0.089-64.85 µmol/g DW leaf. Similarly Merah [33] studied genetic variation among 190 genotypes and found Values of sinigrin content varied from 0 to more than 134 μmol·g−1.

Variability among previous studies is not surprising and can be explained considering first of all the different *B. juncea* varieties used in those studies which also interplay with other factors, such as growing conditions and agronomic practices that can affect plant glucosinolate concentration [34].

Remarkable variation in glucosinolate content from different tissues within one plant may reflect different control mechanism operating on the glucosinolate biosynthetic pathway in different tissues or it may simply result from alterations in substrate availability [20]. The accessions evaluated in the present study were obtained from many different sources. Therefore, the germplasm collection includes variation for growing and storage conditions. Wide variation in glucosinolate content among genotypes, also suggest differences in their health promoting properties and the opportunity for enhancement of their levels through genetic manipulation [35].

Our results provide evidence for large genotypic variability within the studied Indian mustard collection. These results are of interest for industrial uses. Indeed, breeders need to combine agronomic and nutritional traits in the same genotypes. Some studied genotypes showed high levels of sinigrin and could be introduced in breeding programs for improvement of sinigrin content in particular.

Table 1: Genotypes / plant material along with their sources

Sr. No.	Code	Genotypes	Source	Specific Features/ Traits
1.	G1	RC-199	CCSHAU, Hisar	Frost resistant
2.	G2	Purple Mutant	Collected from CCSHAU Germplasm	High lectin
3.	G3	RH-8812	CCSHAU, Hisar	Bold seeded, Suitable for irrigated condition
4.	G4	T-6342	IARI, New Delhi	Tolerant to mustard aphid
5.	G5	Pusa Jai Kisan	IARI, New Delhi	High productivity, Suitable for irrigated conditions
6.	G6	PCR-7	DRMR, Bharatpur	Draught resistant
7.	G7	B-85 glossy	PORS, Behrampur	Glossy plant
8.	G8	RH-7846	CCSHAU, Hisar	Hairy leaf surface
9.	G9	Kranti	CSA, Kanpur	Tolerant to high temperature at seedling stage
10.	G10	Rohini	CSA, Kanpur	Draught tolerant, Suitable for irrigated conditions
11.	G11	Varuna	CSA, Kanpur	Bold seeded, Smooth leaf surface, Thermotolerant
12.	G12	RW white glossy	Collected from IARI Germplasm	White petals
13.	G13	BEC-144	Exotic collection (Polish)	White rust resistant
14.	G14	RW 2-2	Collected from IARI Germplasm	Tolerant to aphid
15.	G15	RLM-514	PAU, Ludhiana	High glucosinolates, High erucic acid
16.	G16	RLM-198	PAU, Ludhiana	High erucic acid
17.	G17	RGN-73	ARS, RAU, Shri Ganganagar	Suitable for growing under irrigated and draught condition
18.	G18	RH-30	CCSHAU, Hisar	Bold seeded, Non shattering
19.	G19	RH-819	CCSHAU, Hisar	Draught tolerant, Thermo tolerant, White rust resistant
20.	G20	RN-393 (Arawali)	ARS, RAU, Navgaon	Suitable for rainfed conditions
21.	G21	Pusa Bold	IARI, New Delhi	Bold seeded, Non shattering
22.	G22	RH-8701	CCSHAU, Hisar	Frost resistant, Suitable for late sown conditions
23.	G23	NPJ-93	IARI, New Delhi	High temperature tolerant at seedling stage
24.	G24	Krishna	GBPAUT, Pantnagar	Higher oil content
25.	G25	CS-54	CSSRI, Karnal	Suitable for salt affect areas
26.	G26	RL-18	PAU, Ludhiana	Salt tolerant
27.	G27	Pusa Jagganath	IARI, New Delhi	Suitable for irrigated condition
28.	G28	Vardan	CSA, Kanpur	Suitable for late sown conditions
29.	G29	RB-50	CCSHAU, Bawal (Rewari)	Suitable for salinity and rain fed conditions
30.	G30	RK-9501	CSA, Kanpur	Shattering resistant, suitable for late sown conditions

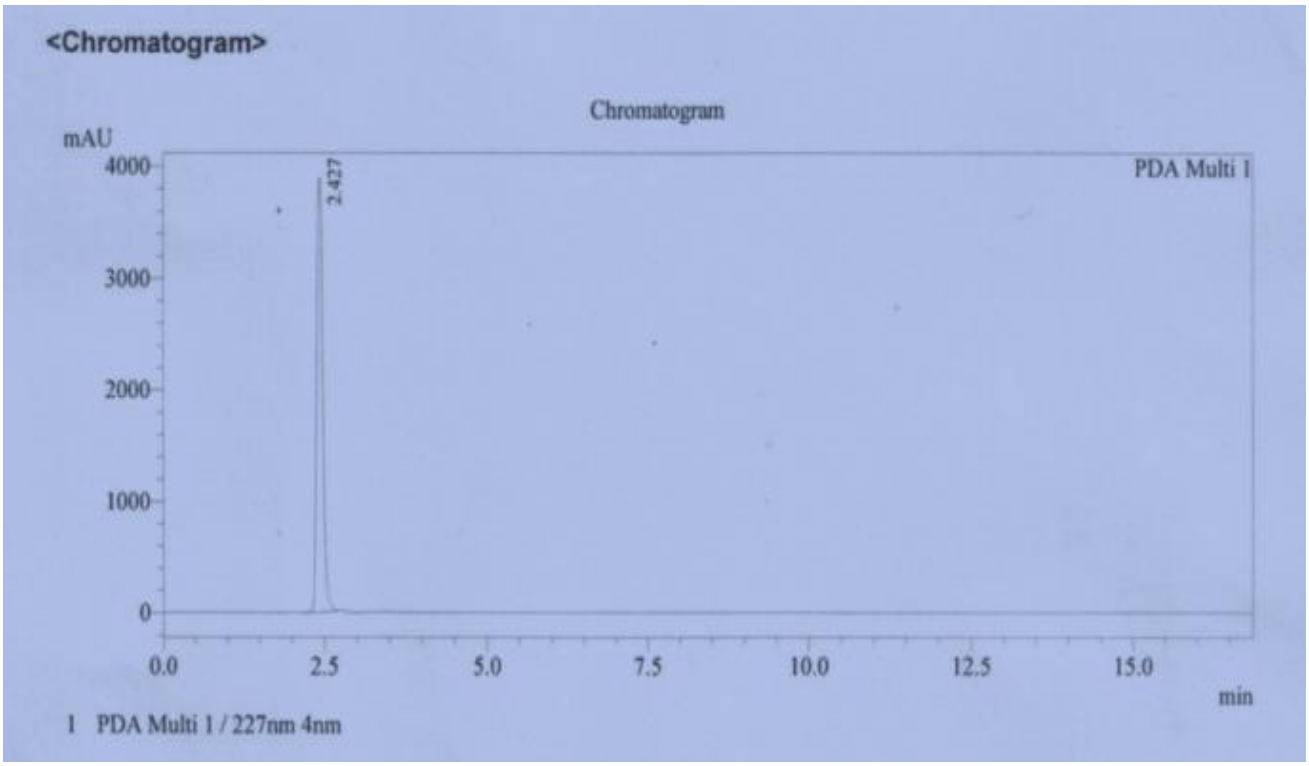

Fig 1: Chromatogram of Sinigrin

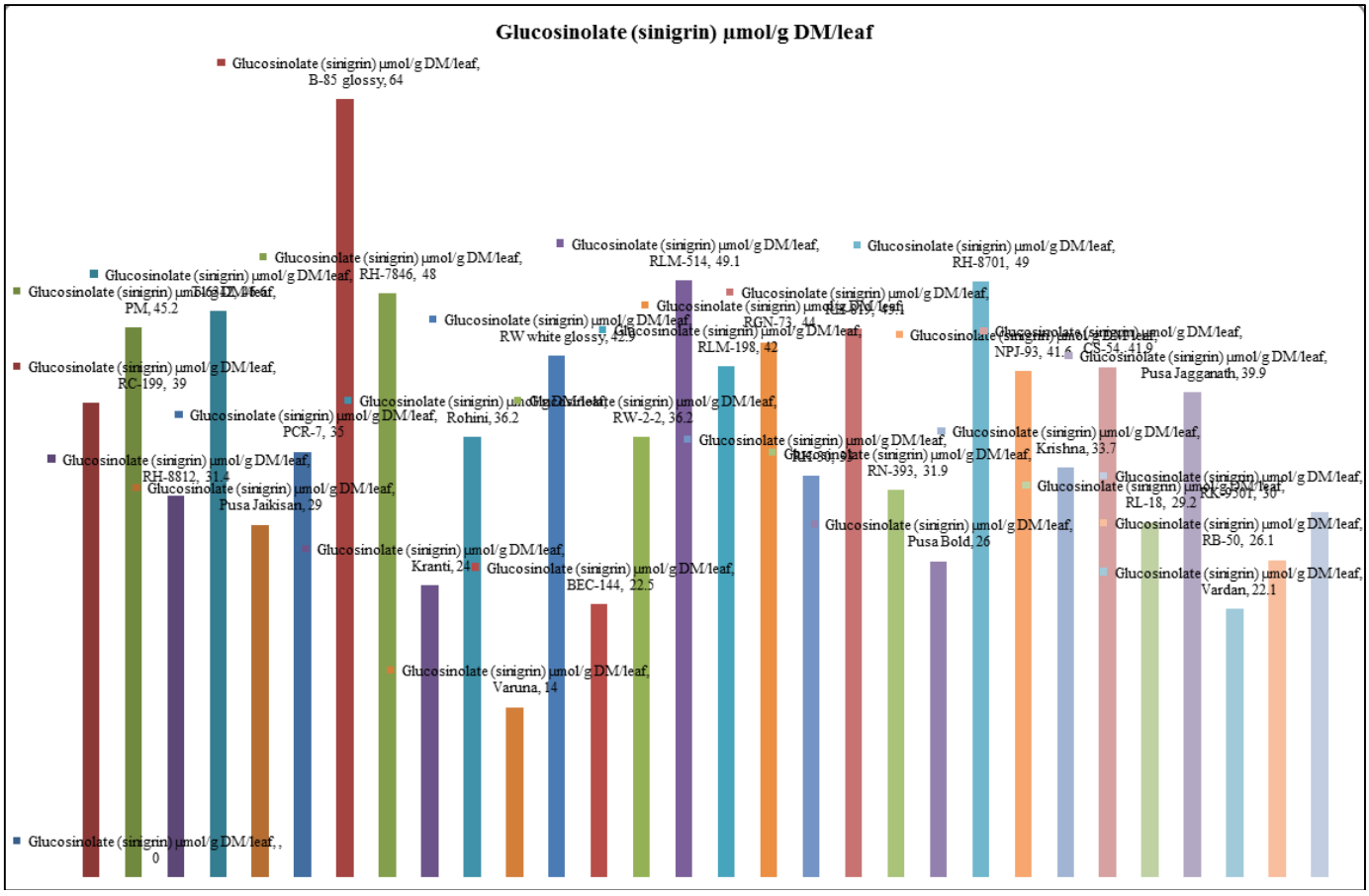

Fig 2: Concentration of sinigrin in leaf of *Brassica juncea*

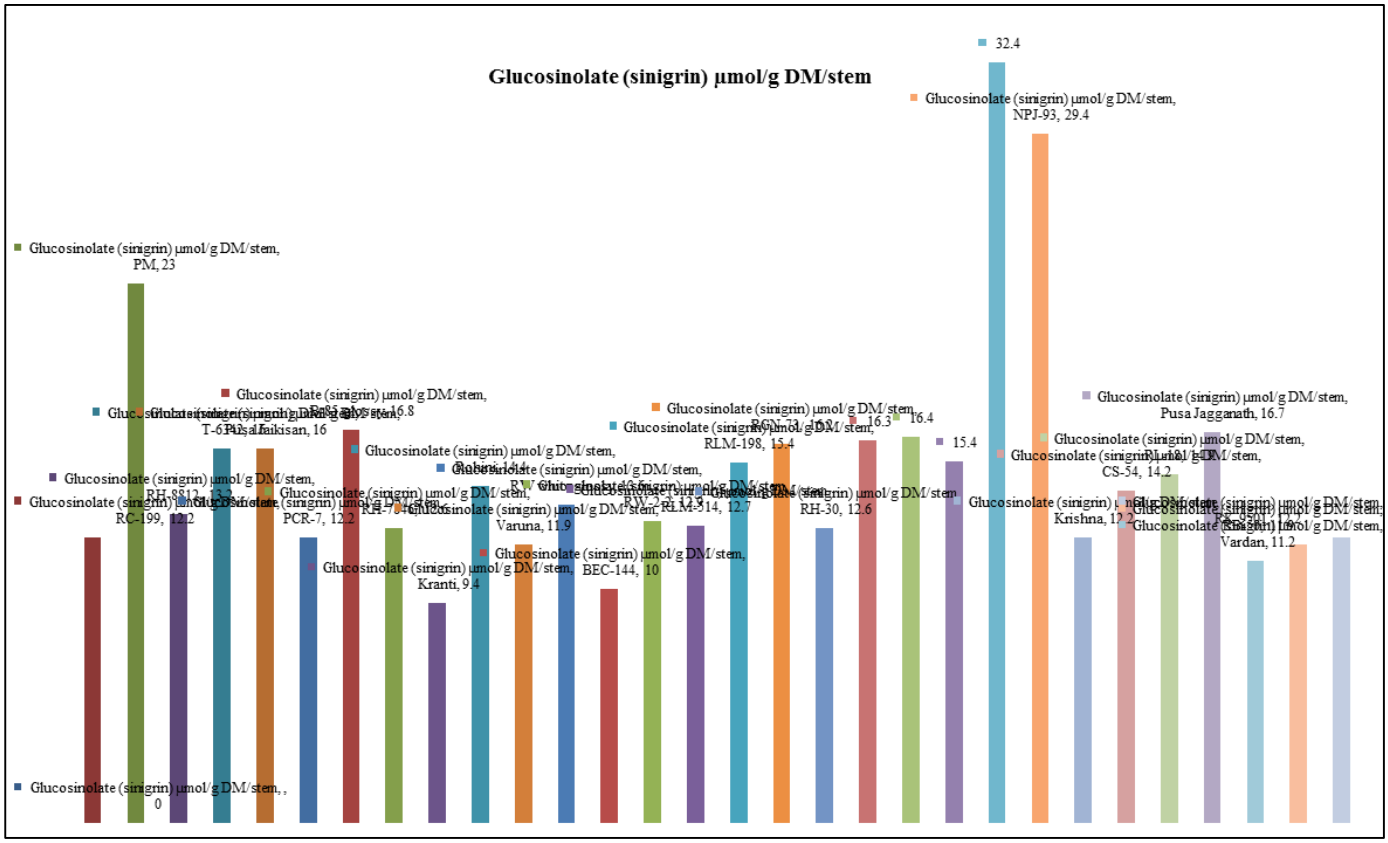

Fig 3: Concentration of sinigrin in stem of *Brassica juncea*

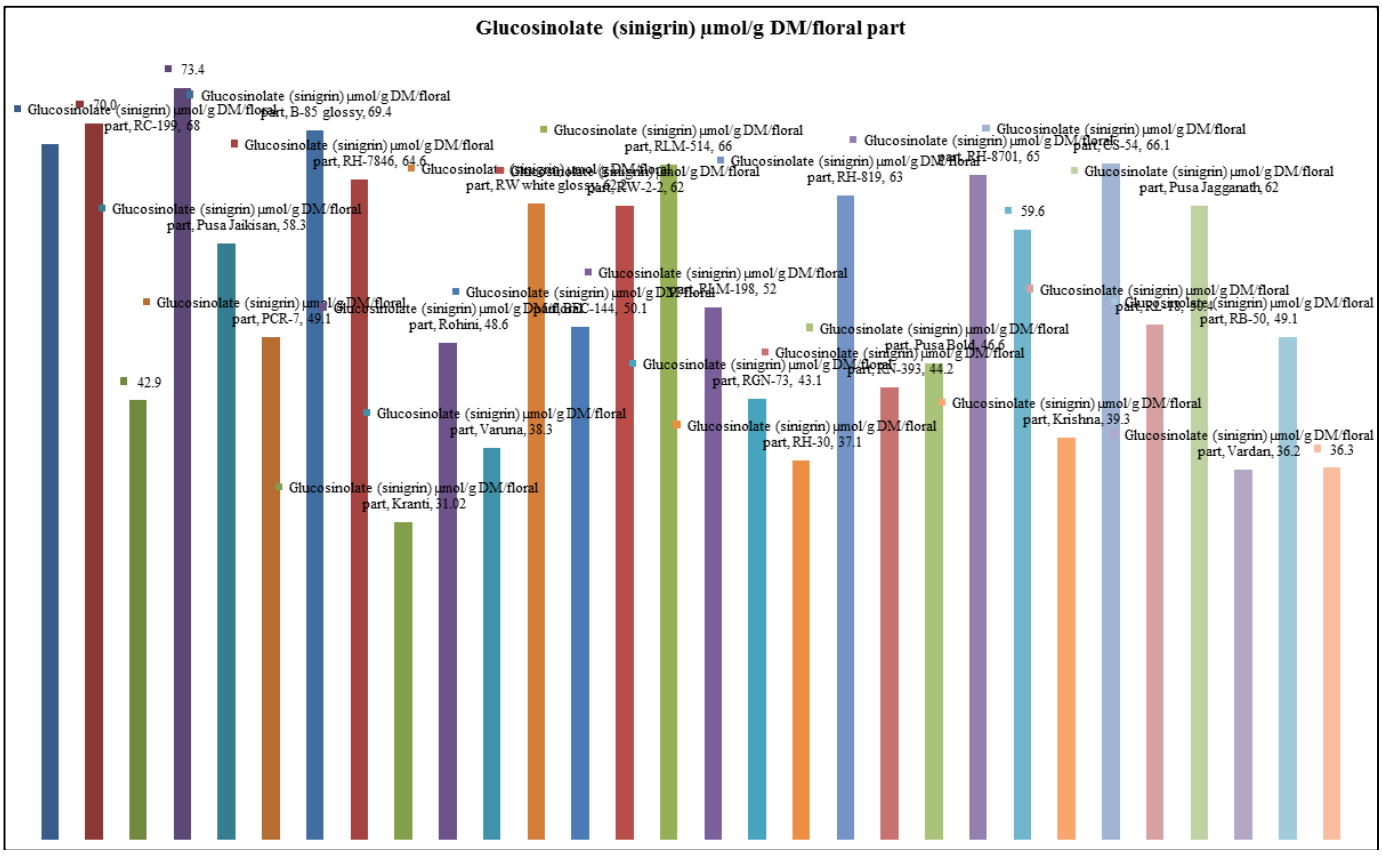

Fig 4: Concentration of sinigrin in floral part of *Brassica juncea*

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