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GLUCOSINOLATES AND OTHER ANTI-NUTRITIVE COMPOUNDS IN CANOLA MEALS FROM VARIETIES CULTIVATED IN EGYPT AND JAPAN

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ABSTRACT

Canola meals from six varieties cultivated in Egypt (Seru4 and Pactol) and Japan (Kirariboshi, Tohoku95, Oominantane and Kizakinonatane) were investigated regarding anti-nutritive compounds, namely glucosinolates, phytic acid, sinapine and total phenols. All varieties except Kirariboshi contained a high level of total glucosinolates. A profile of 12 glucosinolates in all varieties was identified; aliphatic glucosinolates, progoitrin, epiprogoitrin, gluconapin, glucobrassicanapin and glucoberin were the most abundant. Other aliphatic glucosinolates, including sinigrin and gluconapoliferin, were of low level. Indolic and aromatic glucosinolates (4-Hydroxybrassicin, Glucotropeolin, Glucobrassicin, Gluconasturtin and 4-Methoxybrassicin) were low and showed few differences among varieties. The anti-nutritional effects are associated, to a large degree, with the content of aliphatic glucosinolates rather than indole glucosinolates, and the total aliphatic glucosinolates are present at a higher level in all varieties than total aromatic glucosinolate (including indole glucosinolate). Therefore, canola meal from all varieties could cause many anti-nutritive effects. Also it was found that Seru4, Pactol, Tohoku95, Oominantane and Kizakinonatane are high glucosinolates varieties because they contained more than 30 µmol g^{-1} however, only Kirariboshi was a low glucosinolate variety. The differences in the content of individual and total glucosinolates among the varieties may be attributed to genetic and environmental factors. Phytic acid was at a high level in all varieties with few differences. Oominatane contained the lowest level and Pactol contained the highest one. No remarkable differences were observed among Kirariboshi, Kizakinonatane and Tohoku. For total phenols, Tohoku contained the lowest level of total phenols while Pactol and Seru4 contained the highest level. Also, insignificant differences were observed among Kirariboshi, Oominatane and Kizakinonatane in total phenols. Sinapine was found in all varieties with significant differences among varieties. Tohoku and Oominatane contained the lowest levels of sinapine, while Seru4 contained the highest one. Insignificant differences were observed among Kirariboshi, Kizakinonatane and Pactol. Thus canola meals from all varieties must be treated to remove or reduce the aliphatic glucosinolates and other anti-nutritive compounds.

Key words: Canola, glucosinolates, phytate, sinapine, phenols

INTRODUCTION

The presence of some anti-nutrients in rapeseed meal is the most important limitation to its potential as a protein supplement. Among these compounds, glucosinolates, commonly referred to as goitrogens, are a uniform class of naturally occurring compounds found exclusively in the plant kingdom and only in a limited number of dicotyledonous families [1, 2]. All members of the *Cruciferae* family, including rapeseed, contain glucosinolates, and more than 100 different glucosinolates are known [3].

Intact glucosinolates are apparently free of toxicity, but on hydrolysis by an endogenous enzyme, myrosinase (thioglucoside glucohydrolase, E.C.3.2.3.1), presents in the seed and unheated meal, they yield undesirable and potentially toxic products. Some of these products are goitrogenic; others are potentially hepatotoxic, whilst the majority is volatile and strongly pungent and responsible for the 'bite' of mustard, radish and horseradish [4]**.** Other symptoms of the ingestion of large amounts of glucosinolates in animals and poultry include hyper-thyroidism, reduced feed intake and performance, enlarged thyroid gland and reduced levels of circulating thyroid hormones [5].

Another compound, phytic acid, is myo-inositol-1,2,3,4,5,6-hexakis-dihydrogen phosphate. In most seeds it serves as a primary phosphorus and myo-inositol reserve and usually accounts for 60-90% of total phosphorus. It also stores other cations and energy [6]. The binding of phytic acid with minerals is pH dependent and complexes with different cations have varying solubility. Phytic acid also binds protein molecules. Whether directly complexed with phytic acid or through cations, the phenomenon of protein binding leads to decreased solubility, functionality and digestibility of proteins [7]. Binding of minerals with phytic acid also results in their reduced physiological availability [8].

Phenolic compounds are widely distributed in plants, and their presence in the rapeseed meal may contribute to certain undesirable properties including dark color, bitter taste, and astringency. These compounds or their oxidized products can also form complexes with essential amino acids, enzymes, and other substances. Therefore,

they are important factors when considering rapeseed meal as a protein source in food formulation [9].

Among these compounds, sinapine, the choline ester of 3, 5-dimethyl-4 hydroxycinnamic acid (sinapic acid) is the most common of all phenolic esters in canola seeds. It constitutes 1-4 % of air-dried oil-free canola meal [10] and gives a bitter taste to canola meal, making it less palatable to animals [11]. Further, its presence in the diet of certain strains of hens that lay brown-shelled eggs leads to an unacceptable fishy odor in the eggs [12].

The objectives of this study were: (i) to determine the diversity of total glucosinolate content and profiles in some canola varieties from Egypt and Japan and (ii) to determine other anti-nutritional factors including phytic acid, total phenols and sinapine.

MATERIALS AND METHODS

Materials

Six varieties of canola seeds were used in the present study. Two varieties, Pactol and Seru 4, were cultivated in Egypt. These seeds were obtained from the Field Crops Research Institute, Giza, Egypt. Other varieties, Tohoku 95, Oominatane, Kizakinonatane and Kirariboshi (Japanese varieties) were cultivated in Japan and were obtained from the National Agricultural Research Center for Tohoku Region, Japan.

Methods

Preparation of canola meals

One hundred gm of canola seeds (from each variety) were manually cleaned to remove foreign materials and undeveloped seeds, crushed using a household mill (Braun, Germany) and heated to 90^{o}C for 20 min (to inactivate the enzyme myrosinase) in a conventional oven. Then they were defatted by soaking in n-hexane for 48 h with several changes of the solvent. The canola meals were then chemically analysed.

Determination of glucosinolates

Extraction

Two hundred mg of canola meal were transferred to a test tube and heated in a water-bath at 75° C for 1 min. Two milliliters of boiling methanol solution (70% v/v) were added and 200 µl of 20 mmol/internal standard solution of sinigrin (Indofine Chemical Company, USA) were added immediately. The heating at 75° C was continued for a further 10 min, shaking the tube at regular intervals. The tube was centrifuged at 3000g for 3 min and the supernatant was transferred to another tube. Two milliliters of boiling methanol solution were added to the tube containing the solid residue and the tube was reheated for 10 min, and then centrifuged for 3 min, as described above. The supernatant was added to the tube containing the first supernatant and the volume of the combined extracts was adjusted to 5 ml with water.

Purification and desulfation

Pasteur pipettes were placed vertically on a stand and a glass wool plug placed in the neck of each pipette. Half a ml of suspension of ion exchange resin (DEAE Sepharose CL-6B, Pharmacia Biotech, Sweden) was transferred to each pipette. The pipettes were rinsed with 2 ml of the imidazole formate (Wako Company, USA) solution (6 mol) followed with 1 ml portion of water. One milliliter of the glucosinolate extract was transferred to a prepared column and two 1 ml portions of sodium acetate buffer were added. The buffer was drained after each addition. Diluted purified sulfatase (EC 3.1.6.1, Sigma, USA) solution was added to the column (75 µl) and left to act overnight at ambient temperature. The second day, the desulfoglucosinolate was eluted with two 1 ml portions of water and collected in a tube placed under the column. Then the sample was ready for HPLC analysis.

HPLC analysis

The different glucosinolates in canola meal were determined by using High Performance Liquid Chromatography (HPLC) (Hitachi Company, Japan). The desulfoglucosinolates were separated using a type C18 column (CAPCELL PAK C18 Type: C18 AG 120 A Size 4.6 mm × 150 mm, 5 µm) with a flow rate of 0.5 ml/min at 30° C . Elution of desulfoglucosinolates from HPLC was performed by a gradient system of water (A) and acetonitrile/water (25:75, v/v, B). The total running

time was 45 min with a gradient as follows: 100% A and 0% B for 5 min, then in 35 min to 0% A and 100% B and in 5 min back to 100%A and 0%B. An UV detector was used at a wavelength of 229 nm. Individual glucosinolates were identified in comparison with the retention time of siningrin standard. Quantification of individual glucosinolates was accomplished using the response factors as published in the ISO protocol (ISO Method, 1992) [13]. Total and individual glucosinolates are expressed as µmol g−1.

Determination of phytic acid

Phytic acid from 5 grams of canola meal was extracted with 100 ml of 2.4% HCl [14]. The extract was eluted with 15 ml of 0.1 M NaCl followed by elution of phytate with 0.7 M NaCl through AG-X8 anion-exchange resin (Bio-Rad Laboratories, Hercules, CA). After the elution with 0.7 M NaCl, 3 ml extract were pipetted immediately into a conical centrifuge tube and 1 ml of Wade reagent (0.03% $FeCl_3$.6 H_2O and 0.3% sulfosalicylic acid in distilled water) was added [15]. The mixture was centrifuged at 3000 *xg* for 10 min and the supernatant read at 500 nm by using water to zero the spectrophotometer (Shimadzu UV-160A, Japan). The phytic acid content was calculated from a standard curve run using sodium phytate (Wako Company, USA)

Total phenols

Phenolic compounds were extracted from canola meal with 70% aqueous methanol. The total phenolic content of the canola meal was determined according to Folin-Ciocalteu procedure [16]. Since sinapic acid is insoluble in water, all solutions were prepared in methanol/water (1:2 v/v). In a test tube 0.2 ml of the phenolic extract, 1 ml Folin-Ciocalteu reagent (1:10), and 0.8 ml sodium carbonate solution (7.5%) were added. The volume was made up to 10 ml with distilled water. After 30 min the total phenolic content was measured at 765 nm with a spectrophotometer (Shimadzu UV-160A). Sinapic acid (Sigma) was used as a standard compound.

Determination of sinapine

The method of Wang *et al.* [17] was used to determine the sinapine content in canola meal.

Extraction

Canola meal (50 mg) was weighed into a 15-ml screw-top glass test tube, 5 ml of 70% aqueous methanol (v/v) was added, and the tubes were capped and then agitated. The meal was extracted in a water bath at 75 °C for 20 min. After cooling, 5 ml of 70% methanol was added to each tube, and the tubes were centrifuged at 1500 *g* for 10 min at room temperature. The supernatant was used for sinapine determination.

Preparation of CM-Sephadex C25 ion-exchange column

Ten grams of CM-Sephadex C25 resin (Amersham Biosciences, Shinjuku, Japan) was added to 200 ml of distilled water; the resin was allowed to swell for 8 h. The swollen resin was transferred to a 25 × 400 mm glass column and washed with 2 liters of 1 N HCl, followed by water until the pH of the eluate was neutral. The treated resin (0.6 ml) was packed by gravity into a column (8 × 40 mm with a 10-ml reservoir) and settled with water washing to form a gel bed of about 14 mm in height.

Sinapine assay

Clear supernatant (1 ml) was transferred to the prepared CM-Sephadex C25 cation-exchange column. The column was washed with 10 ml of water and eluted with 10 ml of 2 M acetic acid/methanol (1:1 V/V) by gravity. Absorbance of the extracted sinapine was measured at 330 nm against a 2 M acetic acid/methanol (1:1 V/V) blank.

Sinapine content was calculated according to the equation developed by Wang *et al.* [17] as follows:

$$\text{sinapine (mg/g)} = (\text{ABS} - A)/B(V_t/V_e)(V_{es}/W/1000)$$

where:

ABS = absorbance of eluate at 330 nm

A = y-intercept (absorbance) of standard curve

B = slope of standard curve (ml μg^{-1})

V_t = total volume of extract (ml)

V_e = volume of extract applied to column (ml)

V_{es} = elution solvent volume (ml)

W = weight of meal extracted (g), and 1000 = weight conversion factor ($\mu g\ mg^{-1}$).

Statistical analysis

One-way analysis of variance (ANOVA) was carried out by using SPSS software version 13.0 (IBM Company, USA) and Duncan test was used at $P < 0.05$. At least three replicates for each variety were used.

RESULTS

A profile of 12 glucosinolates in 6 varieties of canola meal is shown in Table 1. In Pactol, the predominant glucosinolates were progoitrin (8.8 µmol g^{-1}), epiprogoitrin (6.4 µmol g^{-1}), gluconapin (12.6 µmol g^{-1}), glucobrassicanapin (10.5 µmol g^{-1}), glucotropeolin (2.4 µmol g^{-1}) and gluconastutrin (1.5 µmol g^{-1}). In Seru4, the predominant glucosinolates were glucoberin (13.1 µmol g^{-1}), progoitrin (17.1 µmol g^{-1}), epiprogoitrin (10.1 µmol g^{-1}), gluconapin (17.5 µmol g^{-1}), glucobrassicanapin (16.5 µmol g^{-1}) and gluconasturtin (1.4 µmol g^{-1}). In Kirariboshi, the predominant glucosinolates were progoitrin (4.8 µmol g^{-1}), glucobrassicanapin (7.2µmol g^{-1}) and gluconapin (1.3 µmol g^{-1}). In Tohoku 95, the predominant glucosinolates were glucoberin (50.0 µmol g^{-1}), progoitrin (40.1µmol g^{-1}), epiprogoitrin (20.9µmol g^{-1}), gluconapin (16.8µmol g^{-1}), glucobrassicanapin (13.4 µmol g^{-1}), and glucotropeolin (4.6µmol g^{-1}). In Oominatane, the predominant glucosinolates were glucoberin (10.5 µmol g^{-1}), progoitrin (27.5µmol g^{-1}), epiprogoitrin (11.5µmol g^{-1}), gluconapin (21.7µmol g^{-1}), glucobrassicanapin (12.2 µmol g^{-1}) and gluconasturtin (5.6µmol g^{-1}) . In Kizakinonatane, the predominant glucosinolates were glucoberin (26.5 µmol g^{-1}), progoitrin (34.9 µmol g^{-1}), epiprogoitrin (17.7µmol g^{-1}), gluconapin (23.1µmol g^{-1}), glucobrassicanapin (10.8µmol g^{-1}) and glucotropeolin (5.0µmol g^{-1}).

For other anti-nutritive compounds, Table 2 shows the content of phytic acid, total phenols and sinapine in canola meals. Phytic acid in all varieties ranged from 3.6 to 5.1%, Total phenols was ranged from 1.6 to 2.4% and sinapine ranged from 0.64 to 1.14%.

DISCUSSION

Glucosinolates

A profile of 12 glucosinolates in 6 varieties of canola meal is shown in Table 1**.** The data show significant differences in the content of individual glucosinolates among the varieties. Generally, our results agreed with those of other investigators [18, 19] with minor differences. The first difference was the number of glucosinolates detected in rapeseed meal by using HPLC technique, being 6-11 in those previous studies, as against 12 in our present one. The second difference was the kind of predominant glucosinolates, being progoitrin, gluconapin, 4-hydroxyglucobrassicin and glucobrassicanapin in the former studies. In our study, other additional glucosinolates were detected at a high level, namely glucoberin and epiprogoitrin, in almost all varieties. In another study [20], 4-hydroxyglucobrassicin and gluconapoleiferin were found in a substantial amount in canola meal; however, their level was very low in all varieties except Seru4 and Oominatane. In contrast, the indole types of glucosinolates (glucobrassicin and 4-hydroxy-glucobrassicin) constituted one- to two-thirds of the total glucosinolates in canola. In fact, the antinutritional effects are associated, to a large degree, with the content of aliphatic glucosinolates rather than indole glucosinolates [21]. As shown in Table 1, the total aliphatic glucosinolates are present at a higher level in all varieties (86.5 to 96% of all glucosinolates) than total aromatic glucosinolate (including indole glucosinolate). Therefore, canola meal from all varieties could cause many antinutritive effects in animals fed canola meal.

For total glucosinolates, results indicated that all varieties except Kirariboshi contain a high level of glucosinolates (Fig. 1). According to the canola council, all varieties are high glucosinolates varieties because they contained more than 30 µmol g^{-1}, however, only Kirariboshi was a low glucosinolate variety. The differences in the content of individual and total glucosinolates among the varieties may be due to genetic and environmental factors. Thus Pactol, when cultivated in Egypt, showed a high level of glucosinolates (44.4 µmol g^{-1}). Also, Seru4 (a new Egyptian variety) was a high glucosinolates variety (80.4 µmol g^{-1}).

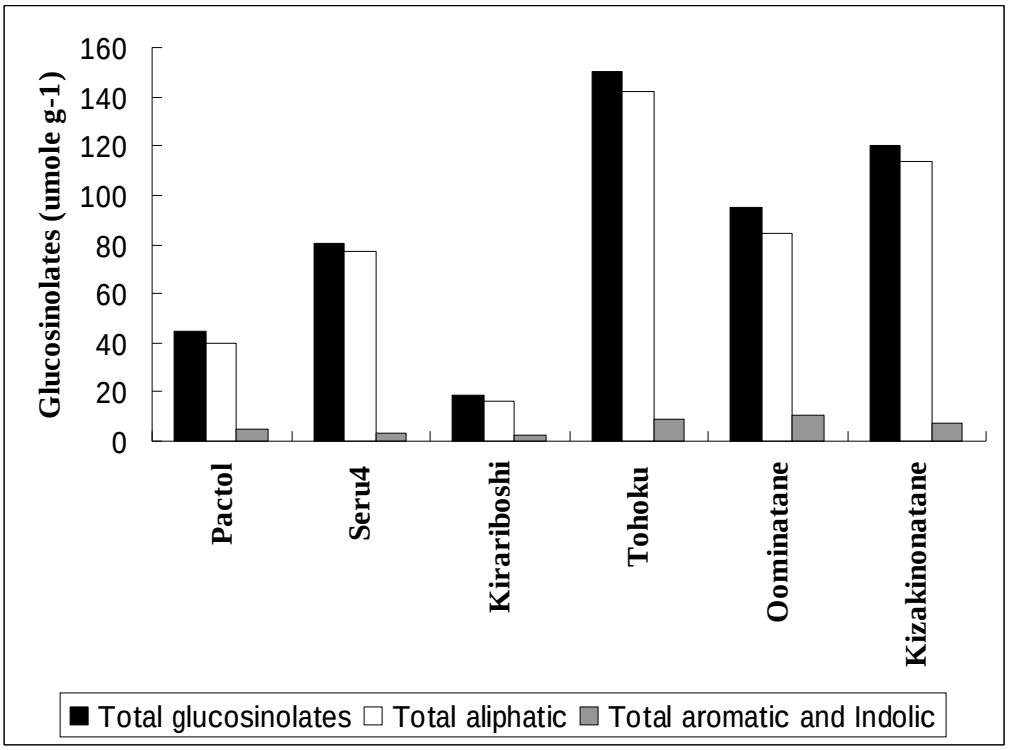

Figure 1: Total glucosinolates, Total aliphatic and Total aromatic and indolic glucosinolates in canola meals

Other anti-nutritive compounds

Phytic acid levels of 2.0-4.0% have been reported for the whole rapeseed [22]. In the present study, a remarkable difference was observed in the level of phytic acid among some varieties. Oominatane contained the lowest level (3.6%), Pactol contained the highest one (5.1%). On the other hand, no remarkable differences were observed among Kirariboshi, Kizakinonatane and Tohoku. Generally, these values are in agreement with those reported by the canola council [23] and other investigators [6, 20] who reported that the defatted meal of canola contained 3.0-6.0% phytic acid. In contrast, it was reported that canola meal contained 2.56% phytic acid [24].

Commonly cultivated rapeseeds, including canola, have similar content of phenolics [25] located mainly in the cotyledons and only trace amounts are found in the seed coats [26]. The content of phenolic compounds in rapeseed meal and flour has been reported to nearly 30-fold that of soybean meal. However, the data on phenolic compounds and tannins in rapeseed meal are fragmentary and diverse [27]. Moreover,

the total content of phenolic compounds in rapeseed meal is reported to range from 1542 mg to 1837 mg per 100 g of defatted meal [28]. In the present study a significant difference among some varieties was observed. Tohoku contained the lowest level (1.6%), while Pactol and Seru4 contained the highest level (2.4%). No significant differences were observed among Kirariboshi, Oominatane and Kizakinonatane. Also, no significant differences were observed among Pactol and Seru4. The levels of total phenols in the present study were higher than those reported by other investigators [9, 24] who found that canola meal contained 0.5-0.64% total phenols.

For sinapine, a notable difference among varieties was observed. Tohoku and Oominatane contained the lowest levels (0.64 and 0.77%, respectively), while Seru4 contained the highest one (1.14%). Also, insignificant differences were observed among Kirariboshi, Kizakinonatane and Pactol. These results lie within the range reported by Fenwick [29] and Bell [20], who found that the level of sinapine in canola meal was 0.6-2.0%. In contrast, another investigator [10] found that the level of sinapine in canola meals was 1.4-4%.

CONCLUSION

All varieties except Kirariboshi were high glucosinolates varieties. Because of the high level of aliphatic glucosinolates and other antinutritive compounds in canola meals from all varieties, some treatments will be needed before using them in the nutrition.

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Table 1: Individual glucosinolate in canola meals (µmol g^{-1})

Glucosinolates	Egyptian varieties		Japanese varieties			
	Pactol	Seru4	Kirariboshi	Tohoku95	Oominatane	Kizakinonatane
Aliphatic						
Glucoberin	0.3±0.10^{a}	13.1±1.25^{b}	0.8±0.20^{a}	50±5.0^{d}	10.5±0.70^{b}	26.5±1.35^{c}
Progoitrin	8.8±1.0^{b}	17.1±0.85^{c}	4.8±0.75^{a}	40.1±1.85^{f}	27.5±1.5^{d}	34.9±0.35^{e}
Epiprogoitrin	6.4±0.55^{b}	10.1±1.05^{c}	0.6±0.25^{a}	20.9±3.05^{e}	11.5±0.05^{c}	17.7±0.30^{d}
Sinigrin	0.0^{a}	0.0^{a}	0.8±0.10^{b}	0.0^{a}	0.0^{a}	0.0^{a}
Gluconapoleiferin	0.8±0.10^{a}	3.2±0.10^{b}	0.5±0.05^{a}	0.6±0.10^{a}	1.1±0.10^{a}	0.6±0.10^{a}
Gluconapin	12.6±0.65^{b}	17.5±0.65^{c}	1.3±0.00^{a}	16.8±3.45^{c}	21.7±2.65^{d}	23.1±0.60^{d}
Glucobrassicanapin	10.5 ±0.20^{b}	16.5±0.10^{c}	7.2±1.55^{a}	13.4±2.85^{b}	12.2±1.7^{b}	10.8±1.4^{b}
Total aliphatic	39.4^{b}	77.5^{c}	16.0^{a}	141.8^{e}	84.5^{c}	113.6^{d}
Aromatic and Indolic						
4 Hydroxybrassicin	0.7±0.15^{a}	0.8±0.10^{a}	0.6±0.25^{a}	0.3±0.10^{a}	0.9±0.10^{a}	0.3±0.10^{a}
Glucotropeolin	2.4±0.40^{b}	0.0^{a}	0.5±0.10^{a}	4.6±1.05^{c}	2.7±0.10^{b}	5.0±0.55^{c}
Glucobrassicin	0.2±0.005^{a}	0.3±0.10^{a}	0.8±0.10^{c}	0.6±0.10^{b}	0.6±0.10^{b}	0.6±0.10^{b}
Gluconasturtin	1.5±0.01^{b}	1.4±0.10^{ab}	0.4±0.10^{a}	2.8±0.45^{c}	5.6±0.75^{d}	1.0±0.25^{ab}
4Methoxybrassicin	0.2±0.00^{ab}	0.4±0.05^{b}	0.2±0.05^{ab}	0.3±0.10^{ab}	0.9±0.15^{c}	0.1±0.05^{a}
Total aromatic and Indolic	5.0^{b}	2.9^{a}	2.5^{a}	8.6^{d}	10.7^{e}	7.0^{c}
Total glucosinolates	44.4±4.05^{b}	80.4±1.15^{c}	18.5±3.2^{a}	150.4±8.0^{f}	95.2±7.45^{d}	120.6±5.7^{e}

Means with different letters in the same row are significantly different from each other ($P < 0.05$)

Table 2: The effect of variety on the phytic acid, sinapine and total phenols content in Canola meals

Variety	Phytic acid %	Sinapine %	Total phenols%
Egyptian			
Pactol	5.1±0.21^{d}	1.04±0.02^{c}	2.4±0.15^{c}
Seru4	4.2±0.06^{b}	1.14±0.06^{d}	2.4±0.15^{c}
Japanese			
Kirariboshi	4.7±0.06^{c}	1.01±0.01^{c}	2.1±0.21^{bc}
Tohoku95	4.9±0.06^{c}	0.64±0.05^{a}	1.6±0.10^{a}
Oominatane	3.6±0.15^{a}	0.77±0.08^{b}	2.0±0.10^{b}
Kizakinonatane	4.7±0.06^{c}	1.06±0.01^{c}	2.1±0.21^{bc}

Means with different letters in the same column are significantly different from each other ($P < 0.05$).

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