

# Screening of Antioxidant Compounds During Sprouting of *Brassica oleracea* L. var. *costata* DC

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**Abstract:** The changes in antioxidant compounds of *Brassica oleracea* L. var. *costata* DC seeds were monitored during the first twelve days of seedling development. Sprouts were screened at time intervals of two days for phenolic compounds and organic acids. The identified phenolic compounds included esters of sinapic acid with glucose, gentiobiose and kaempferol, as well as sinapoylcholine. The organic acids were oxalic, aconitic, citric, pyruvic, malic, shikimic, and fumaric acids. During germination, a depletion of phenolic compounds was observed, although no qualitative changes were seen. Among individual compounds, kaempferol, choline and glucose esters of sinapic acid showed a marked decrease between days two and six, whereas the changes in gentiobiose esters of sinapic acid were smaller. The total organic acids content increased rapidly during the first four days, with less significant variations thereafter. Malic acid, the major organic acid found in sprouts, greatly contributed to this result though oxalic, pyruvic, and fumaric acids also increased in the same manner. In contrast, aconitic, citric and shikimic acids showed decreases between days two and twelve of germination.

**Keywords:** *Brassica oleracea* L. var. *costata* DC, sprouts, phenolic compounds, organic acids.

## 1. INTRODUCTION

The leaves and inflorescences of many Brassicaceae vegetables contribute widely to the human diet, especially in winter. Although Brassicaceae seeds can be used for human consumption as an oil (canola seeds) or mixed with some food products (e.g., bread and cake), sprouts, the germinating form of seeds, are favoured for their nutritional value and have become a familiar component in salads [1-3]. Besides, there is considerable interest in the use of *Brassica* sprouts for health benefits. For instance, sprouts of broccolli can be more effective than mature plants for the prevention of carcinogenesis, mutagenesis, and other forms of toxicity of electrophiles and reactive forms of oxygen [4]. Additionally, it has been reported that germination may reduce the content of anti-nutritional components in the seeds, thus making sprouts safe for the diet [5].

Seed germination is a primary step to generate a new plant. In this process triggered by the imbibition of water, the plant embryo resumes growth after a period of quiescence [6]. The germination process implies a series of active and complex biochemical and physiological reactions, resulting in extensive changes in composition and/or morphology [7, 8]. In fact, during embryogenesis, seeds receive primary

assimilatory products from the mother plant, such as sugars and amino acids, for the synthesis of the storage products: oil (mainly triacylglycerols) and proteins [9, 10]. From the onset of germination (radicle emergence from the seed coat), this reserve is mobilized in order to support growth during early seedling development [11-14]. The presence of glyoxylate cycle and  $\beta$ -oxidation enzymes, especially abundant during post-germination growth, leads to the breakdown of storage lipids to be used directly in respiration and in the synthesis of carbohydrates [11, 15]. The incorporation of these soluble molecules at the level of citrate in the tricarboxylic acid cycle allows the growing of seedlings until photoautotrophic growth occurs. The tricarboxylic acid cycle also provides intermediates for the biosynthesis of various compounds, including amino acids, purines, pyrimidines, haem, and chlorophyll [14].

Phenolic compounds are also involved in plant development during seed germination. In addition, phenolic compounds can also serve in plant-microbe recognition and signal transduction [16, 17]. The phenolics content of sprouts depends on seed quality and the conditions under which they grow. These include temperature, humidity, and the length of germination [18, 19].

The major phenolic compounds found in *Brassica* seeds are esterified phenolic acids [20]. Phenolic choline esters are characteristic and may contribute to the bitter taste and astringency of seed products. Sinapine, the choline ester of sinapic acid, is the most abundant one in seeds of the Brassicaceae family, being considered a major anti-nutritive com-

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pound [2, 21]. The results obtained with *B. oleracea* seed extract showed that phenolics and organic acids in seeds were important as antioxidants [20]. This antioxidant potential can be especially important during germination, when oxygen demand is high [16]. Based on the antioxidant capacity exhibited by the seeds, we can speculate that the presence of these phenolic compounds and organic acids in sprouts might protect the cell from potential oxidation-induced deterioration, thus making this material a good source of antioxidant compounds for human consumption.

The Brassicaceae *Arabidopsis thaliana*, frequently used as a model system in genetics and plant development works, has been studied for regulation of flavonoid gene expression [22, 23] and lipid breakdown [15, 24] in seeds and germinating seedlings. Previous work with *B. oleracea* sprouts concerned the genetic characterization of seed germination [25, 26], monitorization of cell cycle modifications during the first divisions of the seeds [6], sensitivity to hypoxia [18], and the ability of seeds produced under a drought to germinate normally [27]. Other studies have dealt with the ability of seeds to biosynthesize and incorporate erucic acid into triacylglycerols [28] and the activity and distribution of peroxidases isozymes in germinating seeds [29]. The determination of isothiocyanate and glucosinolate content in 3-day-old sprouts of broccoli, and the study of its effects as inducers of enzymes of xenobiotic metabolism in murine hepatoma cells was also carried out [4].

This work is an attempt to establish the relationships among primary and secondary metabolism occurring during the developmental processes of young sprouts, especially in what concerns the changes in organic acids and phenolic compounds. For this purpose, phenolic compounds and organic acids were screened by HPLC/DAD and HPLC/UV, respectively, for a twelve days germination period.

## 2. MATERIALS AND METHODS

### 2.1. Standards and Reagents

Oxalic, malic, shikimic and fumaric acids were purchased from Sigma (St. Louis, MO, USA). Aconitic, citric, pyruvic, ascorbic and sinapic acids and kaempferol-3-*O*-rutinoside were from Extrasynthèse (Genay, France). Hide powder was obtained from FILK (Freiberg, Germany). Methanol and acetic acid were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 2.2. Seeds Germination

*Brassica oleracea* L. var. *costata* DC seeds were collected in 2005 in Bragança, Northeast Portugal. Two hundred seeds were placed on 15 cm diameter Petri dishes lined with fiberglass and watered with 200 mL of distilled water to maintain approximately 100% relative humidity throughout the germination period. The seeds were germinated at 20–23.5°C, under a 16 h-light/8 h-dark regime. A seed sample was used as control. Every 2 days, 5 plates were withdrawn, and germination counts and sprout weight were registered until twelve days of germination. For day 8 only 4 plates and for day 12 just 1 plate were considered.

The harvested sprouts were freeze-dried (Labconco 4.5 Freezone apparatus, Kansas City, MO, USA), ground and stored in a dessicator. Changes of moisture and dry solid during germination were determined after freeze-drying.

### 2.3. Sample Preparation

An aqueous extract was prepared as follows: ~0.5 g of the freeze-dried sprouts were boiled for 15 min in 200 mL of water and then filtered using a Büchner funnel. The resulting extract was lyophilized and kept in a dessicator in the dark. For the determination of phenolic compounds and organic acids, the lyophilized extract was redissolved in water and in sulfuric acid 0.01 M, respectively, and filtered (0.45 µm).

In order to assess possible interferences in the HPLC analysis of phenolic compounds, an analysis of tannins was performed, by treatment with hide powder: 250 µL of redissolved aqueous extract (50 mg/mL) were thoroughly mixed with 2.5 mg hide powder for 1 h and then filtered (0.45 µm).

### 2.4. HPLC-DAD Analysis of Phenolic Compounds

The analysis of phenolic compounds was carried out as reported previously [20] using HPLC (Gilson) and a RP-18 LiChroCART (Merck, Darmstadt, Germany) column (250 x 4 mm, 5 µm particle size), protected with a 4 x 4 mm LiChroCART guard column. Elution was performed using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 30 min and 80% B at 37 min. The flow rate was 1 mL/min, and the injection volume was 20 µL. UV spectra were acquired over the range 200–400 nm using a Gilson diode array detector, and chromatograms were recorded at 320 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. The quantification of phenolic compounds was based on the absorbance in the chromatograms relative to external standards. Since standards of the compounds were not commercially available, sinapic acid derivatives were quantified as sinapic acid and the kaempferol derivatives as kaempferol-3-*O*-rutinoside.

### 2.5. HPLC-UV Analysis of Organic Acids

The separation of the organic acids was carried out as previously reported [30], in a system consisted of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel® Ion 300 OA (300 x 7.7 mm) (Macherey-Nagel, Düren, Germany) in conjunction with a column heating device set at 30°C. Briefly, elution was carried out isocratically at a solvent flow rate of 0.2 mL/min, with 0.01 M sulfuric acid. UV detection was used at 214 nm. Organic acids quantification was based on the absorbance recorded in the chromatograms relative to external standards.

### 2.6. Data Analysis

For organic acids and phenolic compounds quantitation, triplicate experiments were conducted, and the results are presented as mean ± standard deviation. ANOVA followed by Newman-Keuls test ( $p < 0.05$ ) was used to analyse the differences between germination days for phenolic compounds and organic acids.

### 3. RESULTS AND DISCUSSION

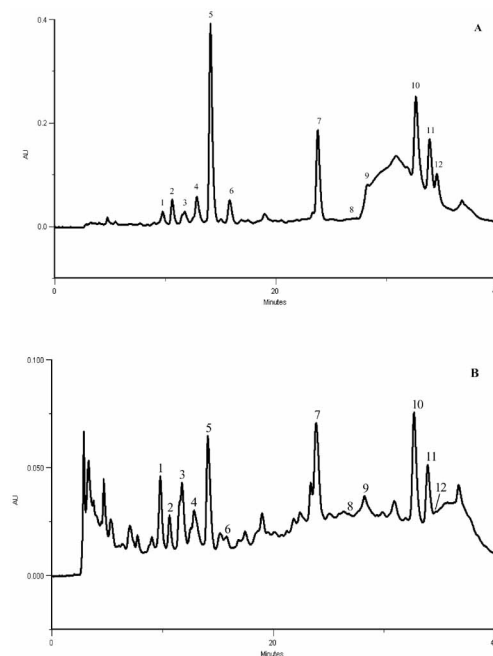
#### 3.1. Seed Viability and Dry Matter Losses During Sprouting

The environment during seed production can have a significant effect on seed performance [18]. Seeds must have 40-60% moisture content for germination to occur and begin to germinate after 24 h of imbibition [29]. On day 2, the seeds had an average moisture content of 66.0%, and 68.3% were already germinated (Table 1). Seed viability was generally high, reaching 81.0% after 4 days of germination. The loss of viability after this period can be attributed to the fact that not all of the germinated seeds were successful in seedling establishment. Over the 12 days development, the sprouts fresh weight increased in a linear manner, from an average of 9.0 mg at day 2 to almost 56 mg by day 12 (Table 1).

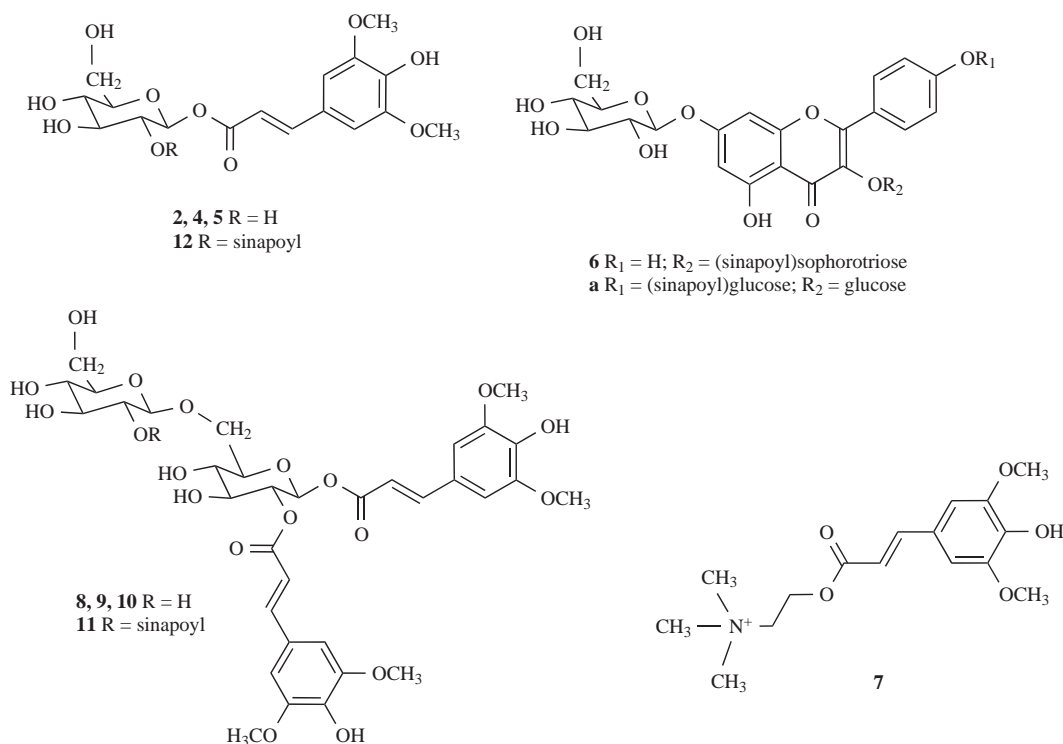
**Table 1. Changes in Sprout Viability, Fresh Weight and Moisture Content with Germination Time**

Germination Time (Days)	Seed Viability (%)	Sprout Fresh Weight (mg)	Moisture Content %
2	68.3±8.52	9.0±0.16	66.0±1.84
4	81.0±6.28	15.6±1.12	79.2±1.61
6	76.6±6.40	23.7±2.37	84.0±1.30
8	71.0±8.57	44.1±6.05	86.7±0.68
10	68.9±4.25	48.8±2.76	89.8±2.07
12	58.7	55.7	91.1

Results are expressed as the means of germination percentages obtained in 5 replicates ± standard error.



**Fig. (1).** HPLC-DAD phenolic profile of *B. oleracea* sprouts aqueous lyophilized extract. Detection at 320 nm. **A)** 2 days germination; **B)** 12 days germination. Peaks: (1) sinapoylgentiobiose; (2) 1-sinapoylglucose isomer; (3) sinapoylgentiobiose isomer; (4) 1-sinapoylglucose isomer; (5) 1-sinapoylglucose; (6) kaempferol-3-(sinapoyl)sophorotrioxide-7-glucoside; (7) sinapoylcholine; (8) 1,2-disinapoylgentiobiose isomer; (9) 1,2-disinapoylgentiobiose isomer; (10) 1,2-disinapoylgentiobiose; (11) 1,2,2'-trisinapoylgentiobiose; (12) 1,2-disinapoylglucose.



**Fig. (2).** Chemical structures of phenolic compounds quantified in *B. oleracea* sprouts. (2) 1-sinapoylglucose isomer; (4) 1-sinapoylglucose isomer; (5) 1-sinapoylglucose; (6) kaempferol-3-(sinapoyl)sophorotrioxide-7-glucoside; (7) sinapoylcholine; (8) 1,2-disinapoylgentiobiose isomer; (9) 1,2-disinapoylgentiobiose isomer; (10) 1,2-disinapoylgentiobiose; (11) 1,2,2'-trisinapoylgentiobiose; (12) 1,2-disinapoylglucose; (a) kaempferol 3,7-diglucoside-4'-(sinapoyl)-glucoside.

### 3.2. Changes in Phenolic Compounds During Sprouting

The phenolics composition of *B. oleracea* L. var. *costata* sprouts was monitored during 12 days of germination. The HPLC/DAD analysis showed the presence of 12 phenolic compounds already identified in the seeds of *B. oleracea*, at all germination times [20]. These compounds include five sinapoyl glucosides, four disinapoyl glucosides, one trisinapoyl glucoside, sinapoyl choline, and one kaempferol glucoside acylated with sinapic acid (Figs. 1,2).

In the chromatogram of sprouts with 2 days of germination (Fig. 1A), a wide hump is present ~30 min, which is characteristic of tannins. The sample treatment with hide powder was not able to remove this, indicating that tannins were not the responsible for the hump.

As it was previously shown for the seeds [20], phenolic acids derivatives were the predominant phenolics found in the sprouts of *B. oleracea* (Table 2). The total phenolics content of sprouts between 2 and 12 days of germination showed significant quantitative changes (Fig. 3): the phenolic compounds were depleted throughout the germination period, having a marked decrease between days 2 and 6. The total amount decreased 85%, from ~11.1 g/kg in sprouts with 2 days to ~1.6 mg/kg in sprouts with 12 days of germination. The decrease in phenolic compounds can be explained by its utilisation for cell wall biosynthesis and as antioxidants [16-17, 23]. Some losses in phenolics can also be explained by the leaching of the water-soluble free phenolic acids upon the imbibition of dry seeds [19].

A seed sample was analysed along with the sprouts to serve as a control. Even though this sample was previously characterised [20], seed quality continues to increase after physiological maturity, with possible changes in phenolic composition during storage time [7, 27]. The amount of phenolic compounds in seeds was ~9.0 g/kg (Table 2), which included 489 mg/kg of kaempferol 3,7-diglucoside-4'-(sinapoyl)-glucoside, a compound with a retention time of 28.5 min, not detected in the sprouts (data not shown). This amount is lower than the average amount found in sprouts with 2 days of germination, although the difference is not significant (Fig. 3).

In what concerns the individual compounds, the glucose esters of sinapic acid showed a significant decrease during the germination period, especially relevant between days 2 and 6 of germination (Fig. 4). In fact, until the 12th day of germination 1-sinapoylglucose isomer (2), 1-sinapoylglucose isomer (4), 1-sinapoylglucose (5) and 1,2-disinapoylglucose (12) decreased between 76 and 98% of their initial content. For kaempferol-3-(sinapoyl)sophorotrioside-7-glucoside (6) and sinapoylcholine (7) the reduction observed corresponded to 96 and 85% respectively.

The decrease of sinapoylcholine (7) in sprouts from 1.7 g/kg on day 2 to 0.3 g/kg on day 12 is a positive feature of the germination process once this compound is considered anti-nutritive [21]. This decrease in sinapoylcholine content can be ascribed to the fact that during the germination of seedlings, sinapoylcholine is hydrolysed to choline, an important substrate in the methylation cycle, and sinapic acid. As a consequence, an increase in sinapic acid would be expected through the germination. However, free phenolic

acids can be polymerized for lignification and structure development on the cell walls of growing seedlings, which may explain the absence of sinapic acid.

Some gentiobiose esters of sinapic acid also decreased significantly, although these changes occurred more gradually within the germination time. Nevertheless, the depletion noticed for compounds 8 to 11, varied between 82 and 92% of the initial amount. Except for compound 8, the decrease of the gentiobiose esters of sinapic acid was <60% on day 10, which might indicate that the germinating seeds preferably use the glucose esters of sinapic acid during germination. The sinapoylgentiobiose isomers (1) and (3) displayed the smallest variations, decreasing less than 25% until the 12th day of germination.

In terms of relative amounts, the phenolic profile of the sprouts showed the presence of 4 major compounds at all of the analysed germination times: 1-sinapoylglucose (5), sinapoylcholine (7), 1,2-disinapoylgentiobiose (10) and 1,2,2'-trisinapoylgentiobiose (11). These compounds accounted for 73 and 68% of total phenolics between days 2 and 12.

The relative amounts of 1-sinapoylglucose isomer (2) (~4%), 1-sinapoylglucose isomer (4) (~6%), 1,2-disinapoylgentiobiose isomer (9) (~2%) and sinapoylgentiobiose isomer (3) (~2%) did not change significantly throughout the germination period, but, sinapoylgentiobiose (1) increased from ~1.4% to 7.4%. On the other hand, kaempferol-3-(sinapoyl)-sophorotrioside-7-glucoside (6) and 1,2-disinapoylglucose (12) decreased from ~7.5% to 2.2% and ~6.6% to 0.7%, respectively. The 2-disinapoylgentiobiose isomer (8) was a minor compound, representing <0.5% of total phenolics.

### 3.3. Changes in Organic Acids During Sprouting

The screening of organic acids belonging to glycolysis, tricarboxylic acid and glyoxylate cycles showed the presence of oxalic, aconitic, citric, pyruvic, malic, shiquimic, and fumaric acids (Figs. 5,6). These acids were previously described in *B. oleracea* leaves and seeds [20, 21, 31], with the exception of pyruvic acid that is now reported for the first time in its sprouts. The total organic acids content increased ~46% from 45.7 g/kg after 2 days of germination to 66.9 g/kg on day 12 (Table 3 and Fig. 7). This may be explained by the increased metabolic activity of the seeds, which rapidly resume the glycolytic and the tricarboxylic acid cycle and the  $\beta$ -oxidation of fatty acids after germination [11, 15].

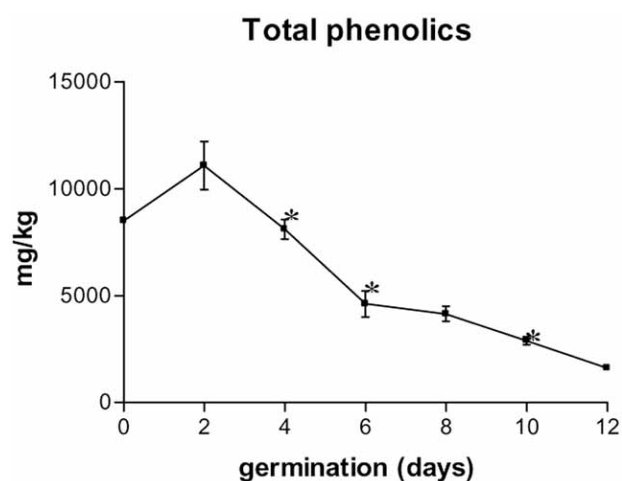
Seeds had an amount of organic acids lower than that present in the sprouts. Ascorbic acid (retention time of 34.2 min), one of the major compounds found in the seeds (~13.3 g/kg, data not shown) was present only in vestigial amounts in the sprouts, being greatly depleted since the beginning of germination, may be due to its well known strong antioxidant effect.

Citric (3) and malic (5) acids were the major organic acids found in sprouts at all germination times (Table 3). Citric acid, accounting for more than 15% of the total organic acids content, decreased ~37% from day 2 to day 12 (Fig. 8). However, despite the significant decrease between days 2 and 4 and increase between days 6 and 8, its variation during

Table 2. Changes in Phenolic Compounds of *B. oleracea* Sprouts with Germination Time (mg/kg, Dry Basis)<sup>a</sup>

Compound	Seed	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
<b>1</b> Sinapoylgentiobiose	240.1 ± 0.44	132.4 ± 12.89 214.1 ± 6.81 158.3 ± 1.11 193.9 ± 0.19 91.4 ± 2.19	189.5 ± 1.52 159.5 ± 10.45 213.7 ± 14.16 186.6 ± 7.85 209.5 ± 4.66	187.7 ± 3.53 144.8 ± 0.74 145.4 ± 3.00 233.5 ± 2.03 140.0 ± 0.40	143.3 ± 3.85 139.4 ± 2.44 187.5 ± 0.04 151.2 ± 9.32	177.2 ± 6.74 143.9 ± 3.24 133.7 ± 2.65 181.3 ± 10.35 161.3 ± 2.09	120.3 ± 6.50
<b>2</b> 1-Sinapoylglucose isomer	381.9 ± 37.00	302.9 ± 3.26 454.3 ± 8.78 317.4 ± 4.40 326.9 ± 22.05 204.3 ± 5.77	288.3 ± 0.19 263.0 ± 1.33 369.5 ± 11.04 304.5 ± 3.31 369.5 ± 2.92	144.4 ± 2.39 99.6 ± 2.45 100.1 ± 1.64 189.3 ± 5.38 117.1 ± 1.28	111.1 ± 0.33 120.8 ± 4.00 188.5 ± 0.63 138.9 ± 10.12	83.8 ± 4.82 72.3 ± 0.51 48.2 ± 1.72 103.6 ± 0.79 78.1 ± 5.51	58.9 ± 0.45
<b>3</b> Sinapoylgentiobiose iso- mer	206.6 ± 23.38	203.6 ± 2.95 280.6 ± 1.47 201.7 ± 0.61 243.0 ± 2.44 119.5 ± 1.54	167.5 ± 1.09 155.0 ± 0.65 174.7 ± 12.08 178.4 ± 27.77 202.8 ± 0.13	221.7 ± 7.02 175.7 ± 3.08 177.0 ± 0.69 194.7 ± 4.35 182.8 ± 3.01	193.6 ± 1.91 173.8 ± 7.05 220.4 ± 3.18 196.4 ± 29.84	180.6 ± 6.80 213.9 ± 15.66 156.3 ± 5.02 232.5 ± 1.13 195.8 ± 9.09	163.3 ± 2.66
<b>4</b> 1-Sinapoylglucose isomer	551.0 ± 19.96	360.2 ± 3.46 476.9 ± 2.91 444.5 ± 1.67 469.5 ± 18.2 269.3 ± 0.58	340.7 ± 0.74 284.5 ± 0.76 355.6 ± 34.45 348.8 ± 5.70 365.2 ± 18.60	184.3 ± 6.47 133.3 ± 8.00 146.9 ± 0.20 209.3 ± 14.71 145.7 ± 4.74	145.3 ± 1.27 200.7 ± 5.15 280.7 ± 6.10 194.9 ± 15.01	144.6 ± 4.95 63.7 ± 3.34 57.5 ± 0.76 97.5 ± 6.68 105.3 ± 6.37	98.3 ± 1.32
<b>5</b> 1-Sinapoylglucose	1320.1 ± 25.34	2810.2 ± 28.73 3505.6 ± 21.79 3036.9 ± 31.88 2779.1 ± 156.34 1254.0 ± 31.38	1614.5 ± 22.28 1596.9 ± 68.77 1946.0 ± 35.29 1684.9 ± 8.87 2020.3 ± 37.21	796.6 ± 20.89 630.9 ± 12.70 581.4 ± 9.35 1168.1 ± 16.39 540.4 ± 0.78	533.1 ± 1.93 575.3 ± 2.55 745.8 ± 6.17 536.3 ± 26.45	210.4 ± 9.86 293.4 ± 8.36 234.7 ± 6.48 333.8 ± 18.59 342.9 ± 9.53	241.5 ± 4.07
<b>6</b> Kaempferol-3- (sinapoyl)sophorotrioxide- 7-glucoside	795.9 ± 13.49	828.4 ± 0.70 990.3 ± 11.98 850.2 ± 11.27 933.7 ± 21.97 544.9 ± 7.97	365.5 ± 1.72 337.9 ± 22.54 424.2 ± 20.89 403.0 ± 11.41 433.5 ± 21.54	119.0 ± 4.23 143.6 ± 1.18 100.6 ± 1.77 180.0 ± 2.96 81.6 ± 2.96	152.4 ± 18.31 239.4 ± 0.80 291.5 ± 17.51 193.6 ± 3.49	103.0 ± 7.23 159.2 ± 14.51 143.1 ± 4.41 167.0 ± 3.40 190.0 ± 21.55	35.9 ± 0.46
<b>7</b> Sinapoylcholine	2064.9 ± 14.55	1608.8 ± 8.91 2606.6 ± 83.56 2356.1 ± 17.53 2606.6 ± 83.56 2408.0 ± 45.12	1535.6 ± 4.53 1482.3 ± 74.67 1882.8 ± 84.71 1720.1 ± 5.59 1811.3 ± 52.66	939.3 ± 84.67 813.7 ± 8.11 790.7 ± 71.64 1074.4 ± 57.36 601.1 ± 42.12	594.4 ± 11.28 764.3 ± 28.17 819.0 ± 19.50 701.6 ± 9.43	322.5 ± 16.04 436.1 ± 5.35 337.1 ± 16.45 375.1 ± 21.02 311.7 ± 0.81	348.5 ± 35.59
<b>8</b> 1,2-Disinapoylgentiobiose isomer	179.6 ± 0.23	48.4 ± 0.12 39.5 ± 0.62 44.0 ± 0.97 42.9 ± 1.57 nq	18.5 ± 0.42 17.3 ± 1.03 23.7 ± 3.41 16.2 ± 4.35 23.0 ± 0.16	4.6 ± 0.55 7.3 ± 0.32 7.1 ± 0.17 4.3 ± 0.14 3.7 ± 0.21	13.6 ± 0.15 12.2 ± 0.40 15.6 ± 0.34 14.2 ± 1.63	11.8 ± 0.11 11.9 ± 0.25 9.7 ± 0.12 9.5 ± 0.83 15.1 ± 0.77	3.7 ± 0.36
<b>9</b> 1,2-Disinapoylgentiobiose isomer	150.5 ± 16.34	229.6 ± 5.30 265.5 ± 9.51 401.5 ± 29.81 266.0 ± 18.44 181.9 ± 5.43	192.1 ± 16.61 93.2 ± 5.15 204.3 ± 9.71 175.8 ± 9.39 181.4 ± 3.83	128.3 ± 4.34 98.5 ± 4.93 49.0 ± 6.08 196.8 ± 1.52 107.0 ± 9.87	76.8 ± 0.09 85.0 ± 1.42 117.9 ± 4.64 165.6 ± 19.40	132.0 ± 1.88 225.5 ± 31.70 110.9 ± 7.08 139.4 ± 9.59 183.0 ± 10.33	30.7 ± 1.06
<b>10</b> 1,2-Disinapoylgentiobiose	1410.7 ± 28.53	1630.5 ± 6.77 2527.7 ± 21.54 2033.7 ± 2.05 2119.8 ± 103.01 884.3 ± 18.12	1259.3 ± 77.14 1078.9 ± 27.46 1514.3 ± 80.47 1584.9 ± 32.08 1685.8 ± 76.75	1152.1 ± 34.16 970.6 ± 15.62 739.1 ± 11.80 1643.8 ± 22.63 851.1 ± 4.74	760.9 ± 27.22 930.7 ± 27.34 1172.0 ± 18.87 847.1 ± 30.48	657.9 ± 13.12 897.2 ± 70.61 587.1 ± 15.04 835.3 ± 29.59 740.5 ± 4.03	329.8 ± 4.21
<b>11</b> 1,2,2'- Trisinapoylgentiobiose	635.4 ± 36.33	1233.1 ± 1.49 1541.2 ± 67.19 1427.6 ± 5.15 1553.3 ± 91.42 710.5 ± 22.09	1062.4 ± 7.50 1013.3 ± 17.34 1269.2 ± 20.37 1331.7 ± 2.47 1427.2 ± 78.57	865.8 ± 32.27 795.6 ± 9.11 587.7 ± 13.95 1361.6 ± 46.43 692.3 ± 4.73	640.7 ± 26.63 700.7 ± 6.87 910.0 ± 19.67 645.2 ± 31.20	570.0 ± 11.23 805.0 ± 26.35 470.6 ± 15.94 594.8 ± 11.47 639.6 ± 17.96	181.3 ± 7.74
<b>12</b> 1,2-Disinapoylglucose	566.7 ± 24.67	585.1 ± 15.92 845.2 ± 10.72 815.3 ± 1.57 791.2 ± 46.72 617.8 ± 0.70	299.1 ± 30.05 296.4 ± 31.59 452.2 ± 6.83 450.8 ± 44.87 455.5 ± 17.47	199.0 ± 5.92 165.9 ± 5.11 117.4 ± 1.32 347.2 ± 4.33 137.9 ± 6.80	99.6 ± 1.63 154.5 ± 9.35 172.4 ± 1.72 165.8 ± 27.47	46.3 ± 1.08 56.3 ± 0.76 38.6 ± 1.07 57.1 ± 3.45 51.8 ± 0.98	181.3 ± 7.74
Total	8503.4	9973.2 13747.4 12087.2 12325.7 7286.0	7332.9 6778.3 8830.2 8386.0 9184.9	4942.9 4179.5 3542.4 6803.1 3600.6	3428.8 4096.8 5121.2 3950.7	2640.2 3378.5 2327.4 3127.0 3015.1	1623.9

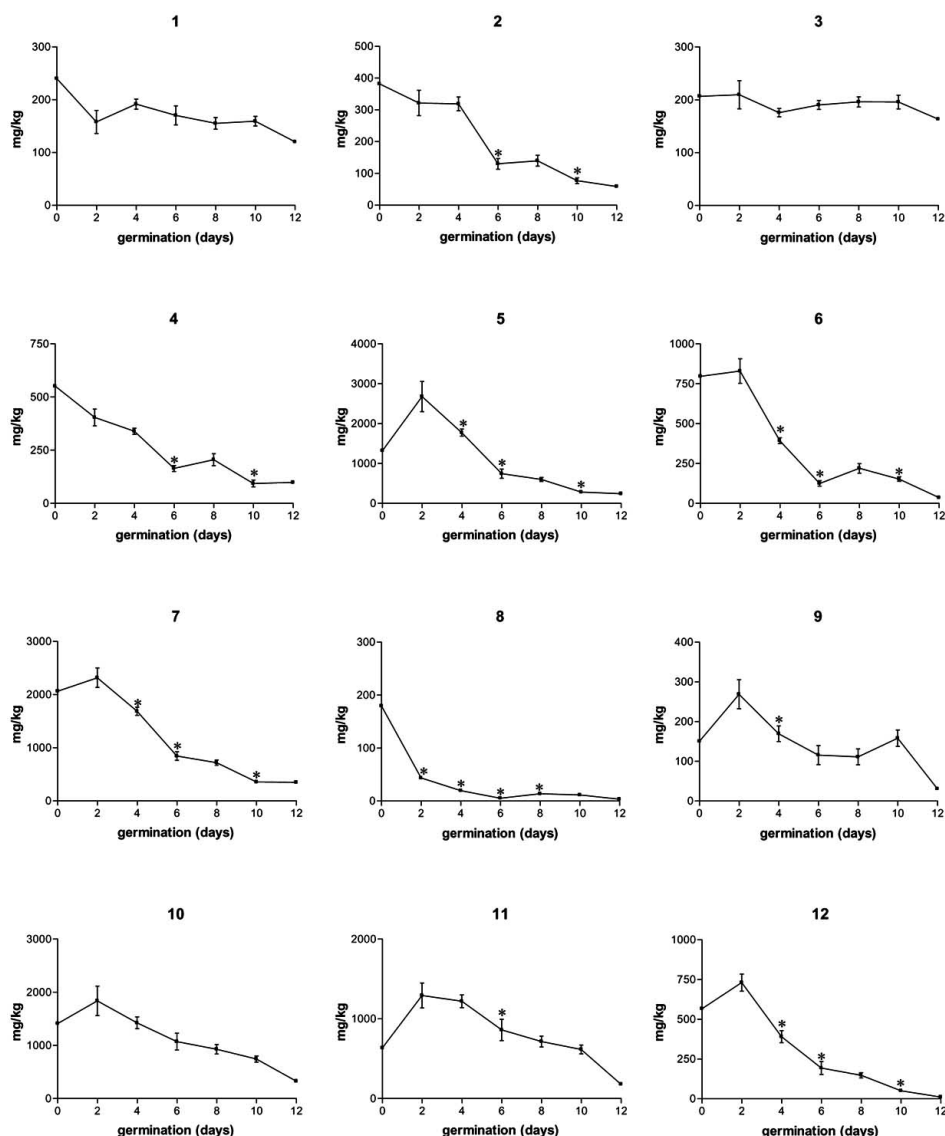
<sup>a</sup>Results are expressed as mean of three determinations ± standard deviation.



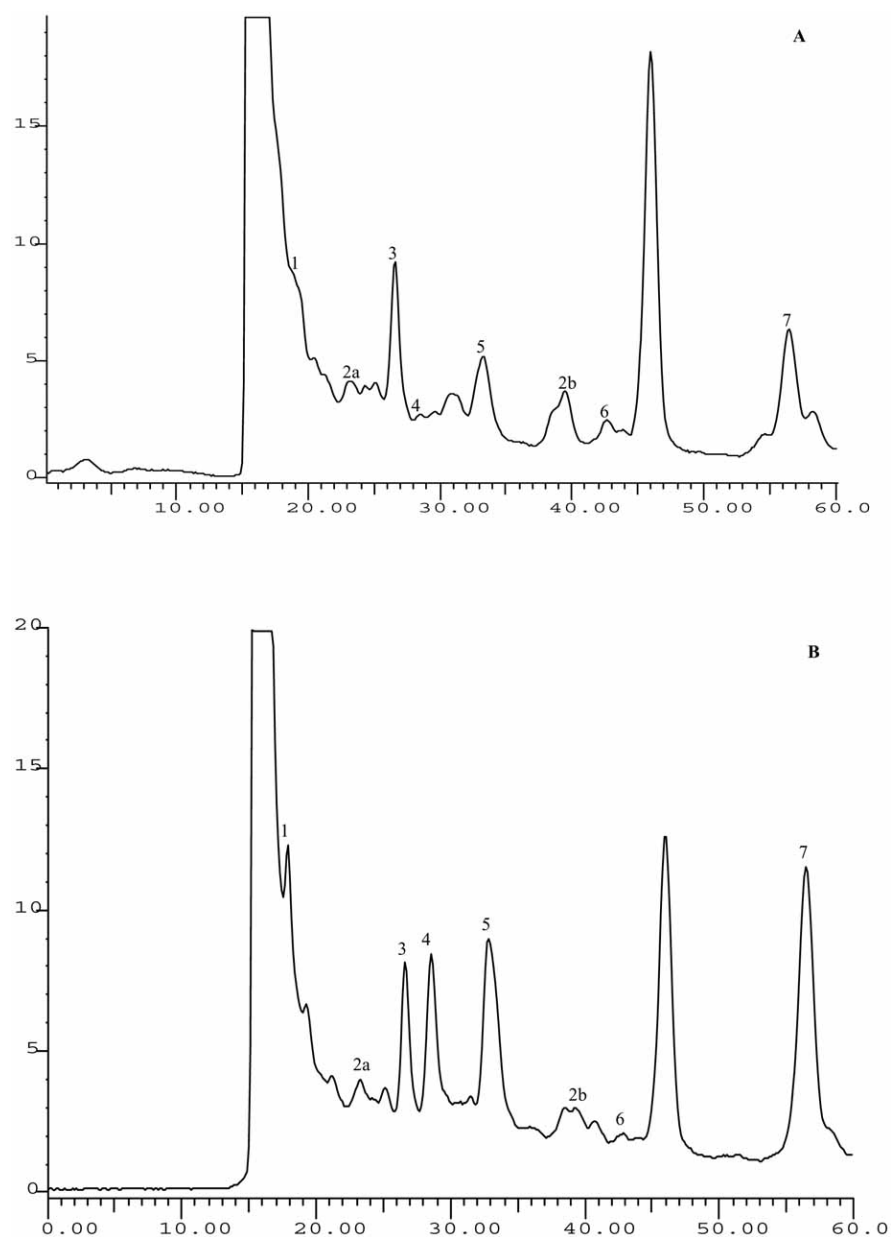
**Fig. (3).** Evolution in total phenolic compounds content of *B. oleracea* sprouts with germination time. \*  $p < 0.05$ , compared with the previous germination time.

the germination period did not show a clear tendency. Malic acid increased from 27.9 g/kg (61% of the total organic acids) on day 2 to 51.6 g/kg (77% of the total organic acids) on day 12, which represents an increase of 85% along the germination period. This was the organic acid that registered the highest increase, which might indicate that besides  $\beta$ -oxidation, the glyoxylate cycle in which fatty acids are converted to sugars having malate as an intermediate product, was active [14].

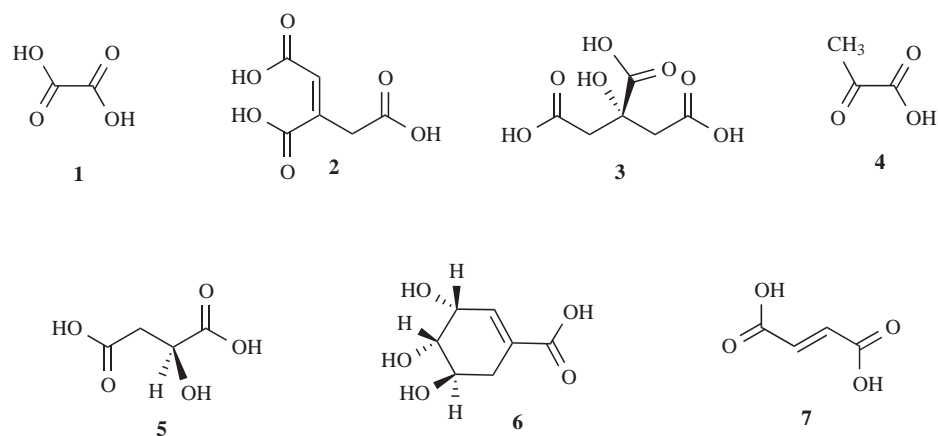
Pyruvic acid (4), which was not detected in seeds and still not quantifiable in sprouts with 2 days, represented ~4% of the total organic acids thereafter. This may be due to its production during glycolysis, as a consequence of the increased respiration rate [13]. The overall amount of oxalic acid (1) did not change significantly. This acid represents less than 4% of the total organic acids at all germination times. Aconitic acid (2), which represented ~0.8% of the total amount of organic acids in seeds, was found to be the minor compound in the sprouts (~0.1%).



**Fig. (4).** Changes in individual phenolic compounds of *B. oleracea* sprouts with germination time. Numbers of panels refer to the number of compounds (see caption of Fig. 1). \*  $p < 0.05$ , compared with the previous germination time.



**Fig. (5).** HPLC-UV organic acid profile of *B. oleracea* sprouts. Detection at 214 nm. **A)** 2 days germination; **B)** 12 days germination. Peaks: (1) oxalic acid; (2a and 2b) aconitic acid isomers; (3) citric acid; (4) pyruvic acid; (5) malic acid; (6) shikimic acid; (7) fumaric acid.

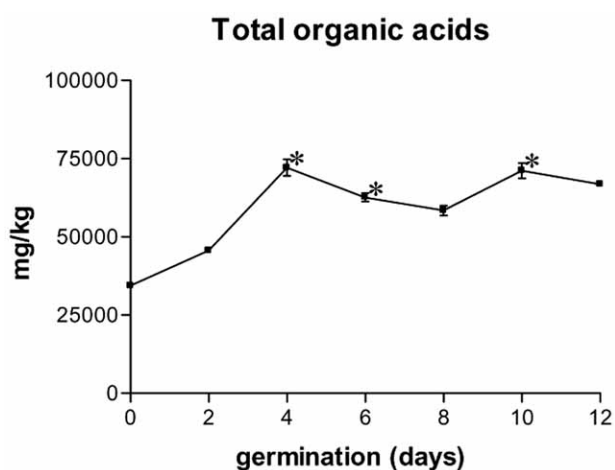


**Fig. (6).** Chemical structures of organic acids quantified in *B. oleracea* sprouts. (1) oxalic acid; (2a and 2b) aconitic acid isomers; (3) citric acid; (4) pyruvic acid; (5) malic acid; (6) shikimic acid; (7) fumaric acid.

**Table 3.** Changes in Organic Acids of *B. oleracea* Sprouts with Germination Time (mg/kg, Dry Basis)<sup>a</sup>

Organic Acid	Seed	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
<b>1</b> Oxalic	821.7 ± 112.31	nq	950.5 ± 49.3	1392.9 ± 29.95	2197.1 ± 74.26	862.0 ± 19.49	1094.4 ± 104.12
		nq	1248.7 ± 96.23	748.6 ± 5.31	1946.0 ± 17.02	2523.2 ± 38.71	
		nq	1740.8 ± 28.31	1419.8 ± 42.44	1122.4 ± 49.19	1960.5 ± 37.94	
		nq	1266.1 ± 7.89	729.9 ± 0.66	2597.2 ± 17.31	2202.2 ± 150.89	
		nq	1724.2 ± 241.64	668.4 ± 4.36	1204.9 ± 25.71		
<b>2</b> Aconitic	275.8 ± 6.73	39.0 ± 2.86	89.4 ± 3.5	43.5 ± 2.80	41.8 ± 0.94	68.3 ± 1.18	37.8 ± 1.53
		25.0 ± 3.09	50.7 ± 10.44	26.8 ± 0.72	27.9 ± 2.81	27.9 ± 1.22	
		13.6 ± 1.63	96.5 ± 12.84	18.4 ± 0.12	120.6 ± 0.37	30.1 ± 2.67	
		15.4 ± 0.45	47.5 ± 0.18	31.7 ± 0.31	23.6 ± 5.42	34.2 ± 5.68	
		34.5 ± 3.13	122.6 ± 10.40	45.6 ± 0.85		32.4 ± 4.32	
<b>3</b> Citric	13334.1 ± 849.9	15686 ± 1622.7	9966.5 ± 27.0	9456.0 ± 682.86	14096.6 ± 624.23	14034.1 ± 344.83	10583.2 ± 553.54
		18435 ± 130.5	11882.4 ± 1311.2	15048.5 ± 443.56	16303.3 ± 79.39	21588.4 ± 101.14	
		17085 ± 6.1	12247.4 ± 403.57	13497.2 ± 91.06	23261.5 ± 180.98	13058.5 ± 178.14	
		17807 ± 24.4	13138.8 ± 311.63	14912.5 ± 447.32	20250.8 ± 367.10	16137.9 ± 1167.13	
		15513 ± 1069.5	15118.9 ± 922.26	9279.7 ± 54.54		16033.1 ± 103.41	
<b>4</b> Pyruvic	nq	nq	1750.2 ± 21.8	2923.1 ± 109.62	1984.8 ± 90.52	2782.9 ± 112.33	2637.5 ± 65.03
		nq	1748.1 ± 32.39	2487.8 ± 83.28	1752.3 ± 3.75	3191.5 ± 278.86	
		nq	2220.3 ± 62.89	3156.0 ± 242.45	2316.4 ± 34.63	2888.2 ± 145.11	
		nq	1999.6 ± 98.48	1946.8 ± 58.40	2624.0 ± 33.33	2908.3 ± 286.89	
		nq	2340.5 ± 245.72	2334.6 ± 174.25		1846.5 ± 49.11	
<b>5</b> Malic	19599.2 ± 289.57	29282.8 ± 2227.04	53854 ± 256.4	51619 ± 2174.6	37302 ± 374.5	48460 ± 3701.1	51579.0 ± 215.03
		24967.2 ± 77.47	56123 ± 771.2	38951 ± 812.4	34979 ± 1213.1	51866 ± 4531.7	
		30442.3 ± 595.07	47944 ± 2280.8	45734 ± 3621.9	34152 ± 1068.2	46936 ± 2358.3	
		24422.5 ± 64.18	59205 ± 2593.3	44356 ± 222.2	34367 ± 511.5	46194 ± 303.3	
		30454.2 ± 107.24	58901 ± 981.0	46895 ± 1267.3		53300 ± 27.8	
<b>6</b> Shikimic	112.4 ± 5.42	222.8 ± 6.57	146.9 ± 0.06	20.9 ± 2.75	18.9 ± 2.48	16.0 ± 0.29	7.0 ± 0.29
		197.3 ± 6.45	145.3 ± 0.08	54.6 ± 1.43	24.4 ± 0.54	115.9 ± 13.0	
		162.6 ± 4.99	205.8 ± 1.58	23.3 ± 3.06	88.8 ± 1.25	110.0 ± 1.46	
		206.1 ± 0.54	169.0 ± 16.20	51.6 ± 3.14	19.8 ± 0.57	78.0 ± 3.38	
		399.7 ± 16.65	121.3 ± 30.52	52.2 ± 1.42		47.9 ± 11.58	
<b>7</b> Fumaric	334.0 ± 4.64	520.4 ± 23.57	928.3 ± 0.82	563.2 ± 28.36	568.7 ± 0.57	2813.1 ± 48.01	915.7 ± 0.72
		579.9 ± 8.42	933.4 ± 3.90	815.0 ± 13.04	504.4 ± 9.23	471.2 ± 23.39	
		487.8 ± 13.24	701.7 ± 7.83	933.9 ± 12.05	1039.2 ± 23.72	670.1 ± 9.82	
		687.6 ± 5.22	647.2 ± 25.74	1232.9 ± 4.75	558.9 ± 1.47	726.4 ± 23.28	
		600.7 ± 0.96	889.6 ± 12.16	1840.3 ± 16.11		631.7 ± 13.14	
<b>Total</b>	34669	45751	67685	66019	56210	69036	66855
		44204	72131	58133	55537	79784	
		48191	65156	64782	62101	65653	
		43138	76473	63262	60441	68281	
		47003	79218	61116		73096	

<sup>a</sup>Results are expressed as means of three determinations ± standard deviation.

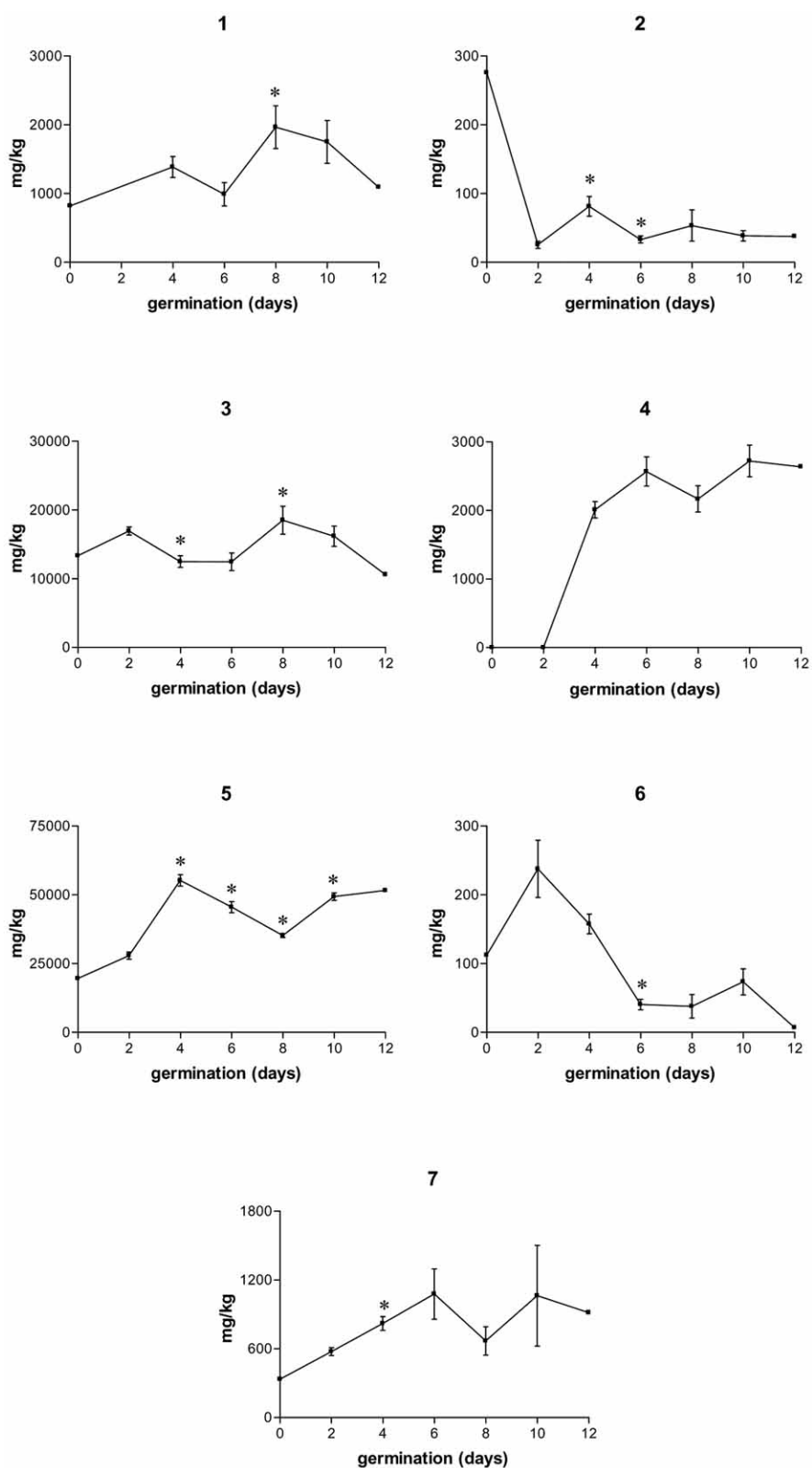


**Fig. (7).** Evolution in total organic acids content of *B. oleracea* sprouts with germination time. \*  $p < 0.05$ , compared with the previous germination time.

As observed with *B. oleracea* leaves and seeds [20, 30-31] shikimic (6) and fumaric (7) acids were present in small amounts, representing less than 0.5 and 2% of total acids, respectively (Table 3). While fumaric acid showed behaviour similar to that exhibited by malic acid, shikimic acid displayed an opposite tendency, significantly decreasing until 6 days of germination.

#### 4. CONCLUSIONS

The screening of the antioxidant phenolics and organic acids during 12 days sprouting of *B. oleracea* var. *costata* revealed that although the identified compounds were the same throughout the germination period, significant quantitative changes occurred, mostly during the first 4 days of germination. While organic acids greatly increased, phenolic compounds had an opposite behaviour. Besides the depletion of the seeds phenolic compounds, the synthesis of the complex kaempferol derivatives, the predominant phenolic compounds in the leaves of mature plants [30, 32], was not accomplished during this germination period. The lack of



**Fig. (8).** Changes in individual organic acids of *B. oleracea* sprouts with germination time. Numbers of panels refer to the number of compounds (see caption of Fig. 5). \*  $p < 0.05$ , compared with the previous germination time.

induction of flavonoid metabolism in the sprouts may be explained by the fact that the available nutrients are being required for the primary metabolism [29].

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**ABBREVIATIONS**

- ANOVA = Analysis of variance  
 HPLC/DAD = High-performance liquid chromatography-diode-array detector  
 HPLC/UV = High-performance liquid chromatography-ultraviolet detector

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