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Full Length Research Paper

Contents of oxalic acid, nitrate and reduced nitrogen in different parts of beetroot (*Beta vulgaris* var. *conditiva* Alef.) at different rates of nitrogen fertilization

Kristina Ugrinović[1], Veronika Kmecl[2], Mirjana Herak Ćustić[3] and Dragan Žnidarčič[4]*

[1]Department of Field Crops and Seed Production, Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia.
[2]Agricultural Institute of Slovenia, Central Laboratory, Hacquetova 17, 1000 Ljubljana, Slovenia.
[3]Department of Plant Nutrition, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10 000 Zagreb, Croatia.
[4]Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.

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Beetroot has many positive effects on human health but can also accumulate considerable amounts of harmful nitrate and oxalate. A two-year open field trial was carried out to evaluate the influence of nitrogen fertilization on the content of different N compounds and oxalate in beetroot. Three nitrogen rates were applied: 0, 75 and 150 kg of N ha^{-1}. Dry matter, oxalate, nitrate N and reduced N were determined in storage root, leaf lamina and leaf petiole. Different parts of beetroot differed significantly in the content of analyzed substances. Leaf lamina had the highest reduced N and oxalate and the lowest nitrate. Storage root usually had the highest nitrate and the lowest oxalate while the reduced N was approximately half of that found in leaf laminas. Contents in leaf petiole were between the leaf lamina and storage root. It can be concluded that different parts of beetroot differ significantly in metabolic activity, with leaf lamina being the most active and storage root the least active part. Nitrogen fertilization had a significant influence on the content of nitrate N and reduced N in all beetroot parts whereas, the contents of dry matter and oxalic acid were, in most cases, not influenced by N rates.

Key words: Fertilization, beetroot, storage root, leaf lamina, leaf petiole, nitrogen, oxalate.

INTRODUCTION

Interest in bioactive compounds found in plant foods especially, fruits and vegetables, has recently increased due to a possibility that they may provide nutritional benefits (Mikulič Petkovšek et al., 2011; Schmitzer et al., 2011; Slatnar et al., 2011). Many classes of bioactive compounds provide benefits beyond normal health maintenance and reduce the risk of illness (Kalt, 2005). Beetroot (*Beta vulgaris* var. *conditiva* Alef.) which is a member of the Chenopodiaceae family has numerous positive effects on human health (Frank et al., 2005; Georgiev et al., 2010) and is often recommended for prevention of the development or occurrence of cancer (Kapadia et al., 2003; Reddy et al., 2003). On the other hand, the nutritive value of beetroot can be reduced if the content of nitrate and/or oxalic acid is high. Amounts of both anti-nutrients can be altered by growing conditions, one of them being nitrogen nutrition (Takebe et al., 1995; Salo et al., 1992; Michalik and Grzebelus, 1995). Studies related to nitrogen nutrition and its effects on the content of nitrate and oxalate in different plants are abundant (Roughan and Warrington, 1976; Rinallo and Modi, 2002; Zhang et al., 2005). Those addressing the relation between accumulations of these substances were mostly done on leafy vegetables while only few

*Corresponding author. E-mail: dragan.znidarcic@bf.uni-lj.si.

Table 1. Meteorogical information, experimental field, Jable (May-September), 2008 and 2009.

Period	Precipitation (mm)			Average temperature (℃)			Insolation (h)		
	2008	2009	Mean[1]	2008	2009	Mean[1]	2008	2009	Mean[1]
May[2]	78.0	95.8	112.3	15.2	15.0	13.2	240.3	231.1	206.4
June	100.7	152.1	151.6	16.6	19.1	16.4	277.6	293.4	217.6
July	89.6	148.9	136.4	19.4	19.4	18.5	279.5	274.8	255.6
August	15.9	186.6	135.5	20.2	18.3	17.7	322.1	225.4	235.4
September[3]	180.3	9.1	121.1	12.1	13.1	14.1	105.0	137.8	115.0

[1]Mean values 1961-2000; [2]After sown; [3]Until harvest.

were published for root crops. Investigations of the relation between the accumulation of nitrate and oxalic acid in beetroot under the influence of different nitrogen fertilizer rates were primarily focused on roots. These relations were not studied in beetroot aboveground organs, with the exception of Goh and Vityakon (1988), who studied them in the root and aboveground parts (leaf petiole and leaf lamina together). However, studies on spinach (Takebe et al., 1995; Elia et al., 1998) showed that leaf laminas and leaf petioles do not respond equally to nitrogen fertilization when accumulation of oxalate is concerned, and therefore should be analyzed separately. Since most of the nitrate in annual plants is reduced in leaves (Darnell and Stutte, 2001), the highest nitrate concentrations are usually found in transport tissues such as stems and leaf petioles (Maynard et al., 1976). It is well known that higher nitrogen rates increase the nitrate content in leafy vegetables (Demšar and Osvald, 2003). Storage roots may have high or low nitrate contents (Maynard et al., 1976). Different studies have shown that in the case of beetroot, higher N rates result in higher nitrate content of storage roots (Salo et al., 1992; Michalik and Grzebelus, 1995). However, nitrate accumulated in cell vacuoles cannot be considered as surplus, because it serves as an alternative to organic osmoticums such as soluble sugars and malate and other organic acids (Blom-Zandstra, 1989).

Synthesis of oxalic acid in plants is associated with N metabolism, notably with nitrate assimilation. To compensate for reduced nitrate, numerous plants synthesize organic acid anions (Marschner, 1995), among which oxalic acid is most common in the goosefoot family (Chenopodiaceae) plants (Nakata, 2003). Proietti et al. (2009), for example, reported that oxalate accumulation in spinach serves to neutralize cytosolic pH alkalized during nitrate reduction. Oxalate can be found in all plant parts. Its concentration is usually highest in leaves and lowest in roots (Franceschi and Nakata, 2005). Oxalic acid content may be influenced by the quantity and form of N added and different parts plants may respond differently to nitrogen fertilizer rates. The amount of oxalic acid may increase, decrease or remain unchanged when increasing the N rate. Takebe et al. (1995) reported that increasing the N rate caused an increase of oxalate in spinach leaf laminas while Elia et al. (1998) detected no such effect. In both cases, the increase of N rate resulted in a decrease of oxalate in spinach petioles. It was reported that nutrition with nitrate N increases the amount of oxalic acid more than nutrition with ammonium N in spinach (Kavazu et al., 2003), beetroot (Goh and Vityakon, 1988), sugar beet (Breteler, 1973), New Zealand spinach (Ahmed and Johnson, 2000) and grasses of the genus *Setaria* (Roughan and Warington, 1976; Goh and Vityakon, 1988).

To further explain, the influence of different nitrogen rates on the levels of oxalate, nitrate N and reduced N in different parts of beetroot (storage root, leaf lamina and leaf petiole), and to establish the relation of the amount of oxalate on the amount of reduced N and the relation between the accumulation of nitrate and oxalate in these beetroot parts.

MATERIALS AND METHODS

Cultural practice

Field trials were conducted in 2008 and 2009 on the experimental field situated at Jable near Ljubljana, Slovenia. The experimental site was situated at 46° 08' N, 14° 34' E and altit ude 315 m. The trials were set up on loam of pH (KCl) 5.4 and humus content between 2.5 and 3.0%. Other soil chemical properties of experimental plots are given in Table 1. Before setting up the experiments, the plots were fertilized with P_2O_5, K_2O and B_2O_3 according to soil analysis (50 kg ha^{-1} P_2O_5, 240 kg ha^{-1} K_2O and 3 kg ha^{-1} B_2O_3 in 2008, and 40 kg ha^{-1} P_2O_5, 240 kg ha^{-1} K_2O and 3 kg ha^{-1} B_2O_3 in 2009, respectively).

The experiments were set up according to a randomized block design with 4 replications. Besides a control treatment without N application, plots were fertilized with 75 and 15 kg N ha^{-1}. Fertilizer in the form of ammonium-nitrate was used as the source of nitrogen. Appropriate amounts of N were divided into two equal parts, of which one was applied at the two-leaf stage and the other at the five to six-leaf stages. The variety Bicores (Bejo Zaden) was sown in May (10th May, 2008 and 8th May, 2009) in rows 30 cm apart. The size of each plot was 3.84 m^2 (1.2 x 3.2 m). When they reached the two-leaf stage, plants were thinned to 8 cm intra-row spacing and to the density of 41.6 plants m^{-2}. Common agricultural engineering practices were applied during crop development (hoeing and protection against (*Cercospora beticola*). Plants from the central part of each plot were harvested at technological maturity in September (20th September, 2008 and 17th September,

Table 2. Chemical properties of the soil of experimental plots, 2008 and 2009.

Parameter	Topsoil, 0-30 cm		Subsoil, 30-60 cm	
	2008	2009	2008	2009
P_2O_5, mg 100 g^{-1}	26.2	46.4	–	–
K_2O, mg 100 g^{-1}	22.3	31.1	–	–
B, mg kg^{-1}	0.35	0.14	–	–
NH_4-N, mg kg^{-1}	2.72	6.1	2.00	*
NO_3-N, mg kg^{-1}	6.46	18.6	3.18	5.88
NO_2-N, mg kg^{-1}	*	*	*	*
Organic N, %	0.18	0.22	0.15	0.16

*Values are under the detection level which is 0.06 mg kg^{-1} of fresh matter for NO_2-N and 0.1 mg kg^{-1} of fresh matter for NH_4-N. Analysis was not performed.

2009).

Climatic data

The data of climate characteristics are from the meteorological station, located in the vicinity of experimental fields. The main meteorological conditions during the investigation period are given in Table 2. According to Bergant et al. (2006) these climatic regimes are very representative of central Slovenia. In both years, average day temperatures during the investigation period were slightly higher than the year average except for September, when they were slightly lower compared to the 30 year average.

Chemical analysis

After being manually harvested, plants of each plot were divided into sub-samples of leaves and roots. Roots were further classified as unmarketable (damaged roots and roots with diameters smaller than 3 cm and greater than 15 cm) and marketable, and were weighted separately. For chemical analysis, 8 to 10 roots with 6 to 9 cm diameters and 10 to 15 fully developed undamaged leaves were selected. Roots and leaves were washed under tap water and air dried. Before the analyses, crowns (root tops) and taproots (root tips) were cut off. Leaves were further divided into leaf laminas and leaf petioles, which were analyzed separately. Dry matter of the finely cut plant samples was determined by drying for 3 days at 60℃ and then, after grinding, for additional 3 h at 105℃. Oxalic acid was determined using a modified procedure of Zarembski and Hodkinson (1962). After homogenization, 5 g of plant sample was mixed with 5 ml of 3 M HCl and kept for 10 min in a water bath at room temperature. Flasks were then made up to volume with 1 M HCl and returned to the water bath for 1 h. After extraction, the samples were filtered through 0.45 µm filter paper. Oxalic acid concentration was determined using an HPLC apparatus (Waters) with a UV/VIS detector at 210 nm. Nitrate was analysed by autoanalysis according to ISO/DIS Standard 14255 (1995). Reduced N (organic plus ammonium nitrogen) was determined by the Kjeldahl method according to the official EC method (ISO 5983, 1997).

Statistical analysis

All analyses were made in duplicate and the results presented as mean values. Data was analyzed using Statgraphics 3.1 for Windows. All analyses were performed at a 95% confidence level. Analysis of variance (ANOVA) was used to test the differences between nitrogen fertilization levels (multifactor ANOVA with fertilization level and replication as factors) and the differences between plant parts (multiple sample comparison). In the case of a positive F-test, the least significant difference (LSD) test was used to compare the means.

RESULTS AND DISCUSSION

Plant growth was affected by unfavourable growing conditions in both years. Since the trial was not irrigated, early plant development was strongly affected by lack of precipitation at the beginning of the growing period in both years. The situation was especially critical in 2008, when precipitation deficiency continued until September. Leaf development was slow and resulted in poor root development. Root yields were therefore at the bottom level of those reported in the literature (Ijoyah et al., 2008). In 2008, the average marketable yield was 10.36 t ha^{-1} and the average total yield was 20.56 t ha^{-1} while average marketable and average total yields in 2009 were 24.33 t ha^{-1} and 29.16 t ha^{-1}, respectively.

Contents of dry matter

Contents of dry matter in different parts of beetroot ranged from 92.08 to 122.50 g kg^{-1} in storage root, from 129.77 to 233.50 g kg^{-1} in leaf lamina and from 78.87 to 113.08 g kg^{-1} in leaf petiole (Table 3). The lowest dry matter content (the average of all three N fertilization levels) was determined for leaf petioles and the highest for leaf laminas. Studies conducted on spinach have also shown that the content of dry matter in leaf laminas is higher than in leaf petioles (Takebe et al., 1995; Elia et al., 1998).

Different levels of nitrogen fertilization generally did not influence the dry matter content in different parts of beetroot. The only exception were storage roots from 2008 when roots from control plots had higher dry matter content than roots from N fertilized plots. Investigations conducted by other authors mostly revealed a decrease of dry matter in beetroot storage root with increasing levels of nitrogen fertilization. In some investigations (Michalik and Grzebelus, 1995), a decrease in dry matter

Table 3. Dry matter content in different parts of red beet according to treatments and years*.

Year	Plant part	Dry matter content, g kg^{-1}				
		Fertilization, kg N ha^{-1}			LSD fertilization(p ≤ 0.05)	Mean
		0	75	150		
2008	Root	103.45^{b}	92.08^{a}	97.80^{ab}	6.91	97.78^{A}
	Leaf lamina	194.93^{a}	233.50^{a}	177.68^{a}	n.s.	202.04^{B}
	Leaf petiole	87.49^{a}	90.66^{a}	78.87^{a}	n.s.	85.68^{A}
LSD plant parts (P< 0.05)						20.59
2009	Root	118.50^{a}	122.50^{a}	115.50^{a}	n.s.	118.91^{B}
	Leaf lamina	129.77^{a}	139.87^{a}	134.74^{a}	n.s.	134.79^{C}
	Leaf petiole	100.92^{a}	113.08^{a}	113.03^{a}	n.s.	109.01^{A}
LSD plant parts (p ≤ 0.05)						8.79

*Within a row (a-b) or a column (A-C) different letters denote significant differences (p ≤ 0.05) between the fertilization levels and plant parts respectively.

Table 4. Oxalic acid content in different parts of red beet according to treatments and years*.

Year	Plant part	Oxalic acid content, g kg^{-1} dry matter				
		Fertilization, kg N ha^{-1}			LSD fertilization (p ≤0.05)	Mean
		0	75	150		
2008	Root	9.02^{a}	9.19^{a}	9.88^{a}	n.s.	9.36^{A}
	Leaf lamina	43.03^{a}	46.35^{a}	55.07^{a}	n.s.	48.15^{B}
	Leaf petiole	50.17^{a}	49.24^{a}	43.01^{a}	n.s.	47.46^{B}
LSD plant parts (P< 0.05)						5.83
2009	Root	8.24^{a}	7.83^{a}	8.79^{a}	n.s.	8.28^{B}
	Leaf lamina	54.17^{a}	46.12^{a}	49.21^{a}	n.s.	49.83^{C}
	Leaf petiole	24.50^{a}	19.33^{a}	18.37^{a}	3.41	20.85^{A}
LSD plant parts (p ≤ 0.05)						4.89

*Within a row (a-b) or a column (A-C) different letters denote significant differences (p ≤ 0.05) between the fertilization levels and plant parts respectively.

was observed at very high nitrogen rates only. Studies on spinach conducted by Elia et al. (1998) showed that increasing the level of N fertilization caused a decrease in the dry matter content of leaf petioles while the dry matter content of leaf laminas did not change significantly.

Contents of oxalic acid

Contents of oxalic acid in dry matter of different parts of beetroot ranged from 7.83 to 9.88 g kg^{-1} in storage root, from 43.03 to 55.07 g kg^{-1} in leaf lamina and from 18.73 to 50.17 g kg^{-1} in leaf petiole (Table 4). In both years, the highest content of oxalic acid was determined in leaf lamina and the lowest in storage root. The same distribution of oxalic acid among plant parts as in our experiments (the highest in leaf laminas, medium in leaf petioles and the lowest in storage roots) was determined earlier for beetroot (Hodgkinson, 1977), sugar beet (Almazan et al., 1996) and spinach (Tanaka et al., 2001). Similar results have been reported also for some other vegetable species. For example, Kmiecik et al. (2004) reported for dill that the content of oxalic acid in leaves alone was much higher than in whole plants. Jaworska (2005) reported that the content of oxalic acid in the leaves of New Zealand spinach was higher than in its stems and leaves together. Different levels of nitrogen fertilization had no significant influence on oxalic acid content in different parts of beetroot, with the exception of leaf petioles from 2009 when the control had higher oxalic acid content than treatments fertilized with 75 and 150 kg N ha^{-1}. Other research reports on spinach indicate that in leaf petiole while the content of oxalate in leaf laminas increases (Takebe et al., 1995) or is not influenced by higher N rates (Elia et al., 1998).

In studies on New Zealand spinach, altering the amount

Table 5. Nitrate nitrogen content in different parts of red beet according to treatments and years*.

Year	Plant part	Nitrate N content, g kg^{-1} dry matter				
		Fertilization, kg N ha^{-1}			LSD fertilization(p ≤0.05)	Mean
		0	75	150		
2008	Root	1.09^{a}	1.76^{a}	3.21^{b}	0.79	2.02^{C}
	Leaf lamina	0.01^{a}	0.01^{a}	0.12^{b}	0.04	0.04^{A}
	Leaf petiole	0.39^{a}	0.61^{a}	2.71^{b}	1.21	1.23^{B}
LSD plant parts (P< 0.05)						0.77
2009	Root	0.63^{a}	1.54^{a}	2.61^{b}	1.13	1.72^{B}
	Leaf lamina	0.01^{a}	0.01^{a}	0.18^{b}	0.16	0.07^{A}
	Leaf petiole	0.50^{a}	0.23^{a}	0.36^{a}	n.s.	0.36^{A}
LSD plant parts (p ≤ 0.05)						0.51

*Within a row (a-b) or a column (A-C) different letters denote significant differences (p ≤ 0.05) between the fertilization levels and plant parts respectively.

of N did not change the content of oxalic acid (Ahmed and Johnson, 2000). The same was also observed by Opiyo (2004) for black nightshade. Since the results of different studies are not congruent, further studies are required to investigate the regulation of oxalate accumulation in different plant parts under different nitrogen fertilization levels.

Contents of nitrate N

Contents of nitrate N in dry matter of different parts of beetroot ranged from 0.63 to 3.21 g kg^{-1} in storage root, from 0.01 to 0.18 g kg^{-1} in leaf lamina and from 0.23 to 2.70 g kg^{-1} in leaf petiole (Table 5). In both years, the lowest content of nitrate N was determined in leaf laminas and the highest in storage roots. These results are within the limits reported in the literature (Brandis, 1985, Salo et al., 1992). Our investigation showed that beetroot leaf lamina accumulates a smaller amount of nitrate than leaf petiole and storage root, which was also reported by Kallio et al. (1982). Studies on spinach showed that the content of nitrate in leaf laminas is lower than in leaf petioles (Takebe et al., 1995; Elia et al., 1998; Santamaria et al., 1999). Kmiecik et al. (2004) reported for dill that its nitrate content in leaves was lower than in stems. Jaworska (2005) reported for New Zealand spinach that stems with leaves contained more nitrate compared to leaves alone. These results support the widely accepted notion that nitrate reduction in annual plants takes place mostly in leaves (Darnell and Stutte, 2001) and therefore, the nitrate content in transport tissues of stems and of leaf petioles is higher than in leaf laminas (Maynard et al., 1976).

It is noteworthy that higher N fertilization levels resulted in higher nitrate N contents in all parts of beetroot, with an exception of leaf petioles from 2009 where no differences between treatments were recorded. Nitrate content generally increased with increasing N rate. Similar results were reported earlier for other crops, for example, for leafy vegetables (Ćustić et al., 1994), for beetroot roots (Salo et al., 1992; Michalik and Grzebelus, 1995; Černe et al., 2000) and the aboveground parts of beetroot (Goh and Vityakon, 1988).

Contents of reduced N

Contents of reduced N in dry matter of different parts of beetroot ranged from 12.65 to 21.80 g kg^{-1} in storage root, from 36.13 to 42.82 g kg^{-1} in leaf lamina and from 15.01 to 20.11 g kg^{-1} in leaf petiole (Table 6). In both years, the highest content was determined in leaf lamina. There was no difference between storage roots and leaf petioles in 2008 whereas in 2009, the content of reduced N in leaf petioles was higher than in storage roots. Almazan et al. (1996) reported for sugar beet that the content of reduced N was the highest in leaf laminas, followed by roots and leaf petioles. In general, the content of reduced N increased with higher N rates, although, the differences between the treatments were not statistically significant for storage roots and leaf laminas from 2009. This supports the opinion that better plant supply of nitrogen increases the quantity of different N compounds (Mengel and Kirkby, 2001).

Conclusion

The influence of different nitrogen rates applied to beetroot was mainly reflected in the content of different N compounds. Higher nitrogen rates resulted in higher nitrate and reduced N in different parts of beetroot while there was no particular influence upon the content of dry matter in different parts of beetroot. Influence of nitrogen fertilization on oxalic acid content was not constant and

Table 6. Reduced nitrogen content in different parts of red beet according to treatments and years*.

Year	Plant part	Reduced N content, g kg^{-1} dry matter				
		Fertilization, kg N ha^{-1}			LSD fertilization(p ≤0.05)	Mean
		0	75	150		
2008	Root	16.34^{a}	19.84^{b}	21.81^{b}	3.36	19.32^{A}
	Leaf lamina	37.01^{a}	38.16^{a}	42.82^{b}	2.74	39.33^{B}
	Leaf petiole	16.36^{a}	17.08^{a}	20.11^{b}	2.14	17.84^{A}
LSD plant parts (P< 0.05)						2.21
2009	Root	12.65^{a}	14.03^{a}	16.41^{a}	n.s.	14.45^{A}
	Leaf lamina	36.13^{a}	38.98^{a}	39.41^{a}	n.s.	38.17^{C}
	Leaf petiole	15.01^{a}	17.58^{b}	18.36^{b}	2.13	17.07^{B}
LSD plant parts (p ≤ 0.05)						1.86

*Within a row (a-b) or a column (A-C) different letters denote significant differences (p ≤ 0.05) between the fertilization levels and plant parts respectively.

therefore needs to be further investigated. Different parts of beetroot differed significantly in their content of dry matter, oxalic acid, nitrate and reduced N. Leaf laminas contained the highest amount of reduced N and oxalate and the lowest amount of nitrate, indicating that these are the most active parts of beetroot plants in nitrate assimilation and metabolism in general. Storage roots usually contained the highest amount of nitrate and the lowest amount of oxalate while the quantity of reduced N was approximately half of that found in leaf laminas. Storage root may be therefore, considered as poorly active in nitrate assimilation and metabolism in general while the role of leaf petiole is somewhere between the leaf lamina and storage root.

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