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Split dose fertilization with urea increases glucosinolate contents in white cabbage (*Brassica oleracea* L. var. *capitata*) under experimental pot conditions

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ABSTRACT

Split dose nitrogen (N) and sulfur (S) applications are a common agricultural practice as safe choices for environmental purposes. However, the effects of split dose practice on glucosinolate (GLS) biosynthesis remain elusive. The objectives of the present study were to investigate the timing effects of N and S fertilizer use on GLS biosynthesis in white cabbage plants. Therefore, timed split and non-split N treatments were combined with three S treatment levels or timed split and non-split S treatments.

Split N dose treatments increased indole and total GLS concentrations, whereas non-split N treatments increased aliphatic GLSs. The effect of S was dependent on N treatment. Split N treatment resulted in enhanced GLS concentrations, which increased from 4351 to 7208 $\mu g\,g^{-1}$ dry weight (DW) with increasing S treatment; and with split and non-split S treatments, GLS concentrations ranged from 5836 to 7208 $\mu g\,g^{-1}$ DW. Non-split N treatment had no effect and GLS concentration was measured at 5510 $\mu g\,g^{-1}$ DW.

Results indicated that equal N availability (split dose) facilitated an increased plant response to S and a subsequent effect on GLS biosynthesis compared to unequal N availability (non-split dose). In terms of practical crop management, the timing of fertilizer addition to white cabbage can be used to optimize GLS concentrations.

1. Introduction

Nitrogen is an essential plant nutrient that plays a key role in plant growth, and crop yield. Crop production practices within the *Brassicaceae* plant family routinely apply S as a standard to optimize cabbage growth for human consumption. Both nutrients serve an important role in the biosynthesis of GLSs, which are the characteristic secondary metabolites found in cabbages and members of the *Brassicaceae* (Verkerk et al., 2009). The enzymatic breakdown products of GLSs are partly responsible for the distinctive taste of cabbage, and also act as a repellent against some herbivores, and attract pollinators (Halkier and Gershenzon, 2006). The GLS structure and chemistry have been thoroughly studied for more than 50 years (Fahey et al., 2001). The GLS investigations have generated great interest due to the health promoting effects of GLS breakdown products. As reviewed by Björkman et al. (2011) and Jahangir et al. (2009), the degradation products were found to reduce developing cancer risks, including colon, bladder, and possibly breast and prostate cancers. Glucosinolate health promoting effects present in *Brassica* species might be influenced by S supply, and the type and form of N fertilizer available during plant growth, because supply has an impact on GLS content and composition (Björkman et al., 2011; Fallovo et al., 2011). These health aspects provide a motive for further investigations of the GLS biology. Glucosinolates consist of a 'thioglucose unit', a 'sulfonated oxime unit', and a variable side chain (Gerendas et al., 2009). The variable side chain is often derived from amino acids, and the GLS group is defined by a specific amino acid. Alanine, methionine, valine, leucine, and isoleucine are aliphatic GLS group precursors; phenylalanine and tyrosine are aromatic GLS group precursors; and tryptophan is a precursor to the indole GLS group (Fahey et al., 2001). Glucosinolates are believed to be localized in the cell vacuole, and accumulated in all vegetative and reproductive plant structures (Brown et al., 2003). In addition, evidence indicates GLSs can be transported through plant phloem (Chen et al., 2001). Glucosinolates can be catabolized by the plant under low S supply to support primary metabolism, *e.g.*, protein synthesis (Falk et al., 2007). Moreover, studies demonstrated that

Abbreviations: N, nitrogen; S, sulfur; GLS, glucosinolate; P, phosphorus; K, potassium; PPFD, photosynthetic photon flux density; DAT, days after transplanting; DW, dry weight; APS, adenosine 5'-phosphosulfate; MYB, myeloblastosis.

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plant N and S uptake was heavily dependent on a sufficient availability of both soil nutrients (Gerendas et al., 2009; Schonhof et al., 2007). In addition, the amount and type of N and S fertilizer available during growth had an effect on GLS concentration in different *Brassica* species, however consistent results were not generated (Fallovo et al., 2011; Omirou et al., 2009). The different N forms are interesting when viewed in light of organic cropping systems, because the fertilizers used are often comprised of different types of N-containing compounds.

Optimizing fertilizer availability throughout a plants' vegetative growth stages is therefore highly relevant to GLS biosynthesis. Optimizing fertilizer availability is standard agricultural practice, and is preferred over a one-dose application. Fertilizer optimization is designed to increase yield and nutrient use efficiency (Kitchen and Goulding, 2001). Optimization in relationship to GLS biosynthesis may likewise be achieved via N and S fertilizers using split-dose treatments, which can ensure a more continuous availability of nutrients over time. However, how this practice affects GLS biosynthesis, or if the timing of fertilizer treatment affects GLS concentrations and composition remains unclear. Therefore, the objectives of this study were to investigate the combined effects of N and S on GLS biosynthesis following one (non-split) or two doses (split) fertilizer treatments during the vegetative growth period in white cabbage, which to our knowledge has not yet been reported in *Brassica oleracea.*

2. Materials and methods

2.1. Pot experiments

Early white cabbage seeds (*B. oleracea* L. var. *capitata* cv. 'Parel') were sown on August 10, 2010 in polystyrene boxes filled with a peat growing medium with a low fertilizer level. The boxes were placed in an unheated greenhouse to germinate. Pots (7.5 L) with saucers were filled with a sandy loam (Typic Agrudalf) field soil sieved through a 5 mm mesh, and mixed with 30% sand (8 kg pot^{-1}, total mass, wet weight). The field soil was taken from the top soil layer (25 cm) at Aarslev, Denmark (55°18'N, 10°27'E) containing 1.35% organic C, 33% coarse sand, 38.8% fine sand, 12.6% silt, and 13.4% clay. The soil/sand mixture contained the following DW chemical composition: pH 7.4 ($CaCl_2$, 0.01 M); 26.2 mg inorganic N kg^{-1}; 2.4 mg inorganic S kg^{-1}; 23 mg P kg^{-1}; and 100 mg K kg^{-1} (P extracted with 0.5 M $NaHCO_3$, K extracted with CH_3COONH_4). Cabbage plants at the 3–4 leaf stage were transplanted to pots, 24 days after sowing, and watered with tap water (2.8 mg inorganic N L^{-1} and 35 mg S L^{-1}) as needed throughout the rest of the experimental period. The plants were grown in an unheated greenhouse, with 50 cm distance among pots, and arranged in a randomized complete block design with four replicates per treatment ($n = 4$). The block design was chosen due to possible spatial variation in the greenhouse conditions. Extra plants ($n = 4$ per treatment) for the harvest halfway through the experiment were grown under the same conditions. During the growing period, the mean temperature was 15.7 °C with 6.6 and 28.5 °C as minimum and maximum temperatures. The plants were provided with artificial light (PPFD 47 μmol m^{-2} s^{-1}) from dawn to dusk, when the outdoor light level did not exceed PPFD 117 μmol m^{-2} s^{-1}. Six days after transplanting (DAT), 1.8 g of N was applied to each pot using granulate urea ($CO(NH_2)_2$, 46% N, Yara, Denmark) to ensure sufficient nutrients for proper initial plant growth. Granulate kieserite ($MgSO_4$, 20% S, K+S Kali GmbH, Germany) was used as an S source. Both nutrients were applied manually to the soil, and watered shortly afterwards. All plants were sprayed with an insecticide (Pirimor) at 12 DAT due to a few cases of aphid attack. At 27 DAT (7–8 leaf stage), the different timing and fertilizer level treatments were initiated. The plants were administered 7.8 g N pot^{-1} in total in the form of urea during the entire experimental period but in different doses and at different time intervals. The following two experiments were conducted simultaneously: Experiment I) split-dose treatment with N (two times 3 g pot^{-1}, applied 27 and 40 DAT, respectively) *vs.* non-split dose treatment with N (one times 6 g pot^{-1}, applied 40 DAT) combined with three different S levels (0, 0.75 and 1.5 g pot^{-1}, applied 40 DAT); and Experiment II) split-dose treatment with S (two times 0.75 g pot^{-1}, applied 27 and 40 DAT, respectively) *vs.* non-split dose treatment with S (one times 1.5 g pot^{-1}, applied 40 DAT). The N combinations in Experiment II were as in Experiment I. Because the highest S level in Experiment I was the same treatment as $S_{non\text{-}split}$ in Experiment II, this provided an overlap in N treatments between the two experiments. Halfway through the experiment, 40 DAT (12–14 leaf stage), one plant per replicate ($n = 4$ per treatment) was harvested for analysis. Soil from two replicate pots representing each treatment 40 DAT was collected after mixing the entire pot soil content. The sample was frozen at −18 °C until analysis. The 2nd treatment was subsequently initiated 40 DAT. At 54 DAT one plant per replicate ($n = 4$ per treatment) was harvested, and one soil sample from each pot ($n = 4$ per treatment), was obtained the following day, using the same method as described above. The total aboveground biomass from each pot was weighed immediately after harvesting, chopped, mixed, and a representative sample of approximately 50 g was immediately frozen in liquid nitrogen, and placed in a freezer (−24 °C) before freeze-drying. After freeze-drying, the samples were ground in a mill, and used for GLS analyses. Immediately after harvest, the remainder of the plant material was oven dried at 80 °C to a constant weight, and DW was calculated. The material was used for N and S analyses.

2.2. Plant and soil analyses

The VDLUFA methods were used to determine plant N and S, and soil NO_3^-, NH_4^+, and inorganic S (VDLUFA, 1976a, 1976b, 1991).

2.3. Glucosinolate analyses

The GLS analyses followed Krumbein et al. (2005), with some modifications. Plant material was extracted with 9 ml 70% methanol (Sigma-Aldrich, Steinheim, Germany) at 75 °C. Following addition of 1 ml 0.4 M barium acetate (Sigma-Aldrich, Steinheim, Germany) and 100 μl 4.5 mM glucotropaeolin (Phytolab, Vestenbergsgreuth, Germany) as an internal standard, the samples were stirred lightly, heated for 5 min at 75 °C, and centrifuged at 12,000 rpm for 10 min. The resulting supernatants were collected, and the pellets extracted two more times with 6 ml 70% methanol at 75 °C, stirred, and centrifuged. The supernatants were pooled, and added to 25 ml 70% methanol; 6 ml was applied to a 500 μl DEAE-Sephadex A-25 ion-exchanger (acetic acid-activated, Pharmacia, Uppsala, Sweden), and washed with 10 ml bi-distilled (Millipore) water. Subsequently, 2 ml 0.02 M acetate buffer and 250 μl purified aryl sulfatase (Sigma-Aldrich, Steinheim, Germany) was applied, and left for 16 h before the desulfo-compounds were eluted with 4 ml bi-distilled water, and filtered through a 0.45 μm Q-Max nylon filter from Frisenette ApS (Knebel, Denmark). Desulfo-glucosinolates were determined by high-performance liquid chromatography on a Dionex Ultimate HPLC 3000 system from Dionex (Germering, Germany) using a Merck LiChroCART Purospher STAR RP-18e column (5 μm, 250 × 4.6 mm), and a 50 μl sample volume. From 0 to 1 min, the mobile phase was 99% purified water and 1% acetonitrile (20%, Th. Geyer, Renningen, Germany). From this point forward, a gradient of 1–99% acetonitrile in purified water was selected from 1 to 21 min followed by 10 min