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Research article

# Preferentially enhancing anti-cancer isothiocyanates over glucosinolates in broccoli sprouts: How NaCl and salicylic acid affect their formation

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ABSTRACT

Broccoli (*Brassica oleracea* L. var. *italica*) sprouts contain glucosinolates (GLs) that when hydrolysed yield health promoting isothiocyanates such as sulforaphane (SF). SF content can be increased by salt (NaCl) stress, although high salt concentrations negatively impact plant growth. Salicylic acid (SA) treatments can attenuate the negative effects of salt on growth. To test whether sprout isothiocyanate content could be elevated without sprout growth being compromised, broccoli seed were germinated and grown for seven days in salt (0, 80 and 160 mM) alone and in combination with 100 μM SA. Increasing concentrations of salt lowered transcript accumulation of GL biosynthetic genes which was reflected in lowered content of Gluconapin, 4-methoxyglucobrassicin and neoglucobrassicin glucosinolates. Other glucosinolates such as glucoraphanin did not alter significantly. Salt (160 mM) increased transcript abundance of the GL hydrolytic gene *MYROSINASE* (*BoMYO*) and its cofactor *EPITHIOSPECIFIER MODIFIER1* (*BoESM1*) whose encoded product directs MYROSINASE to produce isothiocyanate rather than nitrile forms. SF content was increased 6-fold by the 160 mM salt treatment, but the salt treatment reduced percentage seed germination, slowed seed germination, and reduced sprout hypocotyl elongation. This growth inhibition was prevented if 100 μM SA was included with the salt treatment. These findings suggest that the increase in SF production by salt occurs in part because of increased transcript abundance of genes in the hydrolytic pathway, which occurs independently of the negative impact of salt on sprout growth.


## 1. Introduction

Epidemiological and animal studies have shown that consumption of brassica vegetables, particularly broccoli, can prevent coronary heart disease and cancer (Royston and Tollefsbol, 2015). The compounds in the Brassicaceae responsible for these health benefits are the hydrolysis products of glucosinolates (GLs) (Kadir et al., 2015). GLs (thioglucosides) are water-soluble sulfur-rich secondary metabolites that are synthesised from amino acids and glucose *via* aliphatic and indolic pathways. Both pathways have enzymes in common and reciprocally inhibit each other (Gigolashvili et al., 2009). In the aliphatic pathway GLs are mainly derived from chain-elongated methionine, which is first converted to an aldoxime by CYTOCHROME P450 79F1 (CYP79F1) and then to a nitrile oxide by CYP83A1. This nitrile oxide is then modified by a number of enzymes, shared with the indolic pathway, to eventually

*Abbreviations:* GL, glucosinolate; SA, salicylic acid; NaCl, sodium chloride; CYP, Cytochrome; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; *BoMYO*, broccoli myrosinase gene; *BoESP*, broccoli epithiospecifier gene; *BoESM*, broccoli epithiospecifier modifier protein gene; P5CS, delta-1-pyrroline-5-carboxylate; PR-1, pathogenesis related protein 1; GR, glucoraphanin; GI, glucoiberverin; GE, glucoerucin; GS, glucoalyssin; GB, 4-hydroxyglucobrassicin; Mgb, 4-methoxyglucobrassicin; Ngb, neoglucobrassicin; GN, gluconasturtiin; PEITC, 2-phenethyl isothiocyanate; SF, sulforaphane; SFN, sulforaphane nitrile.

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produce the aliphatic core GL structure. In the indolic pathway CYP79B2 converts tryptophan to indole-3-acetaldoxime and CYP83B1 converts tryptophan to indole-3-acetonitrile oxide, which is then modified further by a number of enzymatic steps to the indole core GL structure (Gao et al., 2014). The sulfur required for the final step of core GL biosynthesis is provided by the co-substrate 3′-phosphoadenosine-5′-phosphosulfate (PAPS) produced by ADENOSINE-5′-PHOSPHOSULFATE (APS) KINASE (APSK) of the sulfur assimilation pathway (Sønderby et al., 2010). The importance of PAPS for both the indolic and aliphatic pathways is evidenced by a knockout mutant of APS kinase having substantially reduced GL content (Mugford et al., 2009). Research on *Arabidopsis* has uncovered specific R2R3-type MYB transcription factors as activators of the core GL pathways. Overexpression of *MYB28*, *MYB29* and *MYB76* increases the amount of methionine-derived aliphatic GLs, whereas overexpression of *MYB51, MYB34,* and *MYB122* elevates quantities of tryptophan-derived indolic GLs (Gigolashvili et al., 2009).

GL hydrolysis occurs in damaged tissues because the compounds become exposed to the endogenous enzyme myrosinase. Myrosinase converts GLs to isothiocyanates such as sulforaphane (SF), 2-phenylethylisothiocyanate (PEITC) and other hydrolysis products like indole-3-carbinol and neoascorbigen (Bones and Rossiter, 1996). It is the hydrolysis products of GLs that confer health benefits to humans (Ku et al., 2013b). These chemicals induce human quinine reductase, a cancer phase 2 detoxification enzyme that eliminates carcinogens from the human body (Boddupalli, 2012). The type of isothiocyanate produced by myrosinase depends on which indolic or aliphatic GL is present in the tissue, the tissue pH, concentration of $Fe^{2+}$, and the type of cofactor of myrosinase present, i.e. whether it is epithiospecifier protein (ESP) or epithiospecifier modifier protein (ESM) (Bones and Rossiter, 1996; Nordstrom et al., 2013). It is the relative concentrations of the ESP and ESM cofactors that determines whether nitriles or isothiocyanates are produced (Burow et al., 2008). Under non-stressed conditions the ESP cofactor directs myrosinase to produce nitriles, which are inactive from an anti-cancer perspective (Bones and Rossiter, 1996; Ku et al., 2014). However, in certain circumstances, the ESM cofactor is produced and interferes with the ESP cofactor, thus leading to isothiocyanate production (Burow et al., 2008). Of the reported isothiocyanates, SF is the most potent anti-cancer compound (Matusheski and Jeffery, 2001).

The quantity of GLs in tissues is affected by NaCl, UV radiation, light, nitrogen, sulfur, glucose, fructose, selenium, SA, jasmonic acid, and pathogen attack (del Carmen Martínez-Ballesta et al., 2013; Singh et al., 2015). Researchers have found that NaCl treatments alter GL content in broccoli and radish, with the concentration of NaCl used being critical for whether GLs accumulate or decline. López-Berenguer et al. (2008) increased the total GL content of 20-day-old broccoli seedlings by growing them in 40 mM NaCl and decreased them by growing them in 80 mM NaCl. Guo et al. (2013a) found that 4-day-old broccoli sprouts had higher SF and GR content and elevated myrosinase activity when they were treated with 160 mM NaCl. Guo et al. (2013b) further showed that the GL content in broccoli sprouts was decreased by the lower 20, 40 and 60 mM NaCl treatments. The GL content of seven-day-old radish sprouts was also differentially affected by the NaCl concentration in which they were grown, with lower NaCl concentrations (10 and 50 mM) suppressing total GL content and higher salt concentrations (100 mM) increasing the total GL content of the sprouts (Guo et al., 2013b). The increasing salt concentration also significantly inhibited germination of the radish seed (Yuan et al., 2010). Therefore the mechanism of GL regulation under salinity is complex and still not completely understood (del Carmen Martínez-Ballesta et al., 2013) and the deleterious effects of NaCl on seed germination and plant growth is a substantial problem (Jayakannan et al., 2015).

Salicylic acid (SA) is an endogenous hormone that has an important role in defence against biotic and abiotic stresses (Jayakannan et al., 2015). The hormone has been found to lessen the negative effects of salt stress in tissues (Hayat et al., 2010). For example, SA inhibited the NaCl-mediated reduction in growth of *Brassica juncea* seedlings, in part by enhancing the concentrations of the antioxidant enzymes catalase, peroxidase and superoxide dismutase (Yusuf et al., 2008). The hormone also increased the amounts of proline and improved the photosynthetic capacity of the seedlings by increasing chlorophyll content and carbonic anhydrase activity (Yusuf et al., 2008). Similarly, treatment with SA enhanced the growth of wheat plants under water-deficit stress (Singh and Usha, 2003), and maize, barley, tomato and *Arabidopsis* plants under NaCl stress (El-Tayeb, 2005; Gharbi et al., 2016; Horváth et al., 2015; Khodary, 2004). Young broccoli sprouts are thought to provide greater health benefits than head tissue, because they have much higher concentrations of the SF precursor, glucoraphanin (Fahey et al., 1997).

In this study, we investigated how continuous exposure of broccoli sprouts to various concentrations of NaCl affected sprout growth, transcription and metabolite content of the GL biosynthesis and hydrolysis pathways. We further determined how all of these salt-altered parameters were affected when the sprouts were co-treated with SA, a hormone previously documented to alleviate salinity-induced retardation of plant growth.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Seed of a commercial quality sprouting line broccoli (*Brassica oleracea* L. var. *italica* 'Early Green') were obtained from King Seeds (Katikati, New Zealand). All seeds were surface sterilised according to Chaudhary et al. (2014). For the GL/isothiocyanate measurements and transcript abundance analysis, c. 5 g of seed were sown onto two layers of filter paper in a sterile 15-cm diameter polypropylene tub. The tubs were transferred to a controlled environment chamber with a 16-h-light, 8-h-dark cycle and an air temperatures of 22 and 18 °C, respectively (Avila et al., 2013). Treatments including SA (0 and 100 μM) and NaCl (0, 80 and 160 mM) and combinations were applied by pipetting 5 mL of each solution onto filter paper and then the liquid in each pot was renewed every two days with the solution according to the method of Ozdener and Kutbay (2008) For the GL/isothiocyanate measurements and transcript abundance analysis, the sprouts in each replicate were collected carefully so as not to damage and induce myrosinase activity, frozen in liquid nitrogen and stored at −80 °C. For measurements of growth parameters, 60 seed were germinated in a polypropylene tub with the same treatments. The numbers of germinated seed were counted three times every day at the same time (c. 0900 h, 1300 h, 1600 h) for each of the three replicates. At day seven, 20 whole sprouts were removed from each replicate tub for aerial part (hypocotyl and cotyledons) length and fresh weight measurements.

### 2.2. RNA extraction and quantitative real time PCR

Total RNA was isolated from sprout tissue (ground under liquid nitrogen and stored at −80 °C) using the ZR Plant RNA MiniPrep™ RNA extraction kit (www.zymoresearch.com). The RNA was treated with DNase I (Roche) and cDNA synthesised from 1 μg of the DNase-free RNA with SuperScript III (Invitrogen) and random hexamer primers (Roche). After synthesis, the cDNA was diluted