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Ontogenic Variations of Ascorbic Acid and Phenethyl Isothiocyanate Concentrations in Watercress (*Nasturtium officinale* R.Br.) Leaves

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Watercress (*Nasturtium officinale* R.Br.) is the richest source of glucosinolate nasturtiin, which on hydrolysis produces phenethyl isothiocyante (PEITC). Interest in growing watercress is stimulated since demonstration of the role of PEITC in protection against cancers associated with tobacco specific carcinogens. Twenty-one days old watercress seedlings were transplanted into growth chambers (16-h days/8-h nights of 25/22 °C and photosynthetic photon flux (PPF) of ≈265 μmol m^{-2} s^{-1}). The study was replicated three times. Leaves were analyzed for PEITC and ascorbic acid concentrations at transplant, and harvested at 10-days intervals until 60 days after transplant. The PEITC and ascorbic acid concentrations were the highest in leaves harvested at 40 days and the lowest at transplant. Leaves harvested at 40 days produced about 150% higher PEITC concentrations compared to the leaves at transplant. Both PEITC and ascorbic acid concentrations of leaves increased linearly with age until 40 days after transplant after which there was no significant increase. Seedlings at transplant had the lowest dry mass and leaf area, while plants harvested at 60 days had the highest dry mass and leaf area.

KEYWORDS: Plant growth and development; protected cultivation; mineral nutrition; hydroponics

INTRODUCTION

Cultural conditions including the quality and quantity of light, day length, temperature, and nutritional balance can dramatically influence crop growth and phytochemical yield (*1*). Ascorbic acid (vitamin C) is an important water-soluble vitamin, an excellent reducing agent, and a donor antioxidant in free radical-mediated oxidation processes. It is a widely accepted theory that dietary antioxidants are protective for many chronic diseases, including coronary heart disease (CHD) and cancer. Phenethyl isothiocyanate (PEITC) is a phytochemical that inhibited several types of cancers caused by tobacco-specific carcinogens in rat and mice studies (*2*). PEITC is currently undergoing clinical trials to develop human dietary recommendations. Glucosinolates, the precursors that yield isothiocyanates on hydrolysis, are secondary metabolites produced by crucifers [plants belonging to the family Brasssicaceae, (cruciferae)] as a defense against predation and pest attack. The value of the crucifers in the human diet has been reexamined, since many of the isothiocyanates have been demonstrated to be inhibitors of carcinogenesis in several animal models (*3*). Isothiocyanates inhibited the development of several cancers including cancers of the colon, lung, esophagus, and mammary glands (*2*, *4*−*10*). Phenethyl isothiocyante (PEITC), the hydrolysis product of gluconasturtiin, inhibited cancers in rats and mice that are caused by several tobacco-specific carcinogens (e.g., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitrosomethyl benzylamine, benzo[*a*]pyrene, and *N*-nitrosobenzylmethylamine) (*11*, *12*). PEITC acts as both a blocking agent and an inhibitor of tumor initiation via inhibition of cytochrome P450 enzymes and by induction of phase II enzymes such as glutathione *S*-transferases (*13*).

Watercress (*Nasturtium officinale* R.Br. Brassicaceae) is the most abundant source of gluconasturtiin, with 5.32 g of gluconasturtiin/100 g of defatted seeds, among the crucifers studied (*14*). One hundred grams of fresh watercress leaves contained 43 mg of vitamin C, 4700 IU of vitamin A (*15*), and 34 mg of α-tocopherol (*16*). Isothiocyanate concentrations in radish (*Raphanus sativus* L.) were influenced by day length (*17*) in rutabaga (*Brassica napobrassica* Mill.), and by season of cultivation (*18*) in turnip (*Brassica rapa* L.) cultivars. Previously, it was demonstrated that cultural conditions such as photosynthetic photon flux (PPF), photoperiod, temperature, and nutri-

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tional balance can dramatically influence crop growth, development, and the PEITC concentrations in watercress (*19*−*22*). The objective of this study was to examine the effect of stage of harvest on the ascorbic acid and PEITC concentrations of the leaves at transplant and harvested at 10 day intervals until 60 days after transplant.

MATERIALS AND METHODS

Plant Material. Twenty-one days old watercress (Johhny's Seeds, Albion, ME) seedlings were transplanted into 500-cm^3 square pots containing the commercial medium Metro 510 (O. M. Scotts, Marysville, OH), a mixture of sphagnum peat, vermiculite, and composted pine bark. Forty-eight pots were transferred to the growth chamber (model G-10; Environmental Growth Chamber, Chagrin Falls, OH), equipped with cool-white fluorescent/incandescent light programmed to maintain 16-h days/8-h nights, and temperatures of 25/22 °C.

Plant Growth Environment. Within the chamber, the plants were arranged in four trays with 12 plants in each tray. The PPF (400−700 nm) in the chamber was greatest in the center row of the chamber and lower along the sides of the chamber. The gradient was 325 μmol m^{-2} s^{-1} at the center and 255 μmol m^{-2} s^{-1} along the sides of the chamber. To ensure that all the plants received similar PPF during the study, the plants in the chamber were moved everyday incrementally from the center of the chamber in a continuous cycle across the light gradient. The mean PPF was ≈265 μmol m^{-2} s^{-1}. The mean PPF value reported in this study was obtained by averaging the light measurements taken at various locations in the growth chamber every 3 days throughout the study period. The study was replicated three times as a completely randomized blocks design. Plants were fertilized with N at 50 mg mL^{-1} for the first week and then twice weekly with N at 100 mg mL^{-1} until the last harvest using 20N−4.36P−16.6K water soluble fertilizer (The Scotts Co., Marysville, OH).

Data Collection and Analysis. The plants were harvested at 10-day intervals after transplant until 60 days after transplant. At each stage, fully mature leaves from the third, fourth, and fifth nodes (counting from the shoot tip) were harvested from six plants, and analyzed for PEITC and ascorbic acid. At each harvest, two plants were randomly chosen to obtain the leaf area, leaf fresh weight, and dry weight. All data were analyzed by the Statistical analytical systems (*23*).

Chemical Analysis. The watercress leaves were analyzed for ascorbic acid and phenethyl isothiocyante concentrations at transplant, and harvested at 10-day intervals until 60 days after transplant.

Phenethyl Isothiocyante. *Plant Extraction and Analysis.* The PEITC extraction and analytical procedures were adopted from that of Gil and MacLeod (*24*). One gram whole leaf tissue samples were ground in 10 mL of distilled water and filtered through the funnel into large test tubes. Five milliliters of dichloromethane (DCM) with internal standard (100 ng of phenyl isothiocyanate, catalog no. 13974-2, Aldrich, Milwaukee, WI) was added. The DCM phase was taken into smaller test tubes (that would fit the centrifuge rotor) and centrifuged for 10 min. Another 5 mL of DCM was added and the sample was shaken well. The DCM phase was transferred to smaller vials and 1−2 μL was injected into a gas chromatograph (GCQ GC-MS system, Thermo Finnigan, Austin, TX). Two samples were analyzed for each treatment replicate.

Chromatographic Conditions. For the analysis of PEITC, a capillary column (0.25 mm ID, Phase ratio-125, HP-Innowax fused silica capillary column; length, 30 m; film thickness, 0.5 micron) was used. The transfer line temperature was 230 °C, the ion source temperature was 205 °C, and the injection port temperature was 190 °C. The air and helium flow rates at the detector end were 300 and 35 mL min^{-1}, respectively. The carrier gas (nitrogen) flow rate was 1 mL min^{-1}. Split injection with a split ratio of 50:1. The temperature program had an initial temperature 50 °C (held 1 min) and was increased to a final temperature of 200 °C at the rate of 20 °C min^{-1}. The analysis time was ≈20 min. The peaks were identified using a standard (2-phenyl ethyl isothiocyanate, P 2179, Sigma, St. Louis, MO), and quantified using an internal standard (phenyl isothiocyanate, P 13974-2, Sigma, St. Louis, MO) analyzed under identical chromatographic conditions (**Figure 1**). The peak heights were used instead of the peak area to determine the concentrations of the PEITC in watercress leaves, because of lower variability observed when using peak heights compared to peak areas. A standard curve was constructed and the PEITC concentration was calculated using the formula $c = \mathrm{RPH}/K$ where c = concentration, RPH = the relative peak height, and K = the slope of the curve.

Ascorbic Acid Analysis. The ascorbic acid was analyzed using the 2,6-dichlorophenol indophenol visual titration method described in the official methods of analysis by the Association of Official Analytical Chemists (*25*), and the ascorbic acid concentration was determined per gram of fresh leaf weight.

RESULTS

Plant Material. The plant growth characteristics of the seedlings at transplant, and plants harvested at 10-day intervals until 60 days after transplant are summarized (**Table 1**, **Figure 2**). The plants grown in the specified photoperiod (16-h days/8-h nights), day/night temperatures (25/22 °C), and PPF of ≈265 μmol m^{-2} s^{-1} light intensity did not flower until final harvest at 60 days after transplant. The total fresh weight, total dry weight, fresh leaf yield, dry leaf yield, and the total leaf area were the highest in plants harvested at 60 days and the lowest at transplant (**Table 1**).

Chemical Analysis. The PEITC and ascorbic acid concentrations were the highest in the leaves harvested at 60 days and the lowest in the leaves at transplant (**Table 1**). The leaves harvested at 40, 50, and 60 days produced ≈150, 181, and 195% higher PEITC concentrations, respectively, compared to the leaves at transplant. There was no significant increase in the PEITC concentrations in the leaves harvested at 50 and 60 days compared to the leaves harvested at 40 days after planting.

The ascorbic acid concentration was the highest in leaves harvested at 40 days after transplant and the lowest in leaves harvested at 60 days after transplant. The leaves harvested at 40 days after transplant had ≈69% higher ascorbic acid per gram of fresh weight compared to the leaves at transplant. Both the PEITC and ascorbic acid concentrations of the watercress leaves increased linearly with age until 40 days after transplant after which there was no significant increase in the production of PEITC. However, the ascorbic acid concentration was lower in leaves harvested later than 40 days after transplant.

DISCUSSION AND CONCLUSIONS

Glucosinolates are secondary metabolites, and variability in the secondary metabolite concentrations in plant produce harvested at different stages has been reported earlier in several cruciferous crops. Fahey et al. (*26*) reported that 3-day-old broccoli (*Brassica oleraceae* var. *italica*) sprouts were ≈20 times higher in concentration of glucoraphanin (the glucosinolate precursor of 4-methylsufinylbutyl isothiocyanate or sulforaphane) compared to mature broccoli heads. However, it is to be noted that these studies compared the isothiocyantes concentrations in different plant parts: the sprouts and the flower heads (broccoli heads) harvested at different developmental stages of the plant, the actively growing vegetative stage, and the reproductive stage, respectively. It is previously reported that different organs of the plants contain different ratios of the glucosinolates, and the regulation of these ratios are due to "some sort of metabolic control" at the glucosinolate level (*27*). Results of this study provides evidence for the existence of variability of isothiocyanates in watercress leaves harvested at

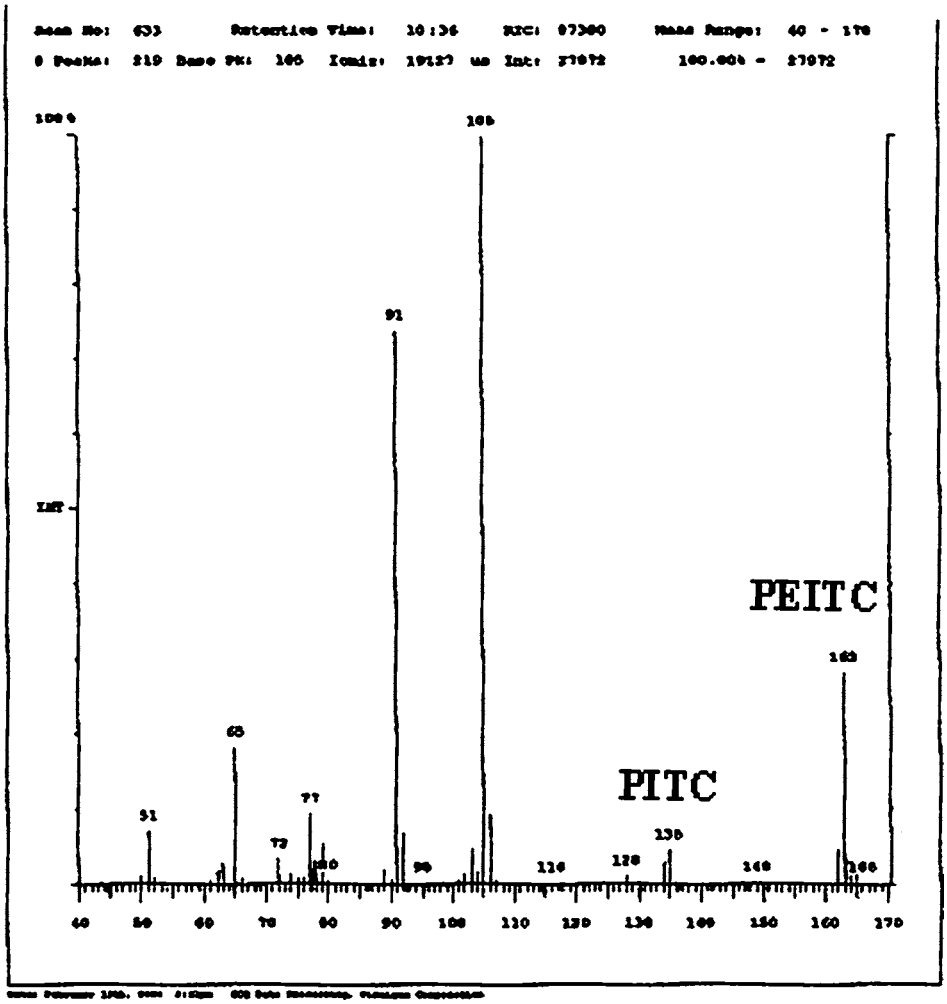

Figure 1.

Table 1. Effect of Stage of Harvest on Growth Parameters and Phenethyl Isothiocyanate (PEITC) and Ascorbic Acid Concentrations in Watercress Leaves[a]

stage of harvest (days after transplant)	PEITC (μg g^{-1} leaf fresh weight)	ascorbic acid (μg g^{-1} leaf fresh weight)	plant height (cm)	leaf yield fresh weight (g)	leaf yield dry weight (g)	leaf area (cm^2)	total fresh weight (g)	total dry weight (g)
0	233 c	371 c	6.4 d	0.03 f	0.001e	0.39 f	0.07 f	0.003 d
10	257 c	407 c	12.4 c	0.67 f	0.01 e	35.3 f	1.37 f	0.03 d
20	389 b	413 c	30.2 b	3.27 e	0.19 d	165.7 e	5.8 e	0.30 d
30	453 b	509 b	46.1 a	13.70 d	0.89 c	263.1 d	19.6 d	0.77 cd
40	590 a	626 a	45.0 a	21.66 c	2.83 b	666.7 c	30.6 c	1.44 c
50	654 a	257 d	48.3 a	29.67 b	3.13 a	755.7 b	52.6 b	4.95 b
60	688 a	187 e	50.3 a	36.73 a	3.54 a	952.0 a	100.1 a	9.76 a

[a] Mean separation by Duncan's multiple range test ($P \leq 0.05$). Means followed by similar letters not significantly different.

different ages. The concentrations of PEITC in watercress leaves increased with age at which the leaves were harvested, with no further increase when harvested later than 40 days after transplant.

The ascorbic acid concentrations increased linearly from the day of transplant until the leaves were 40 days old and then declined at later stages of harvest. Unlike the PEITC, which is a secondary metabolite, ascorbic acid is an important component of the plants with multiple roles participating in many plant processes including photosynthesis, antioxidant, photoprotection, cell wall growth and expansion, resistance to environmental stresses, and synthesis of ethylene, gibberellins, anthocyanins, and hydroxyproline (*28*). Thus, it is reasonable to speculate that ascorbic acid synthesis and hence its concentrations increased linearly during the active growing period of cell wall growth and expansion as reflected in the increase of plant height, up to 40 days after transplant; thereafter, the ascorbic acid concentrations decreased, perhaps due to decreased synthesis by way of reallocation of the carbon for the production of other compounds and/or greater use in the various oxidative processes associated with aging of the plant and metabolic processes associated with entering the reproductive phase.

Data on the growth characteristics showed a linear increase in plant height, leaf area, and fresh and dry weight yield. It is apparent that the metabolic pathways of ascorbic acid and PEITC do not follow the general trend of fresh and dry mass production (**Figure 3**). Although higher fresh (70%) and dry (25%) leaf yields were obtained from the plants harvested at

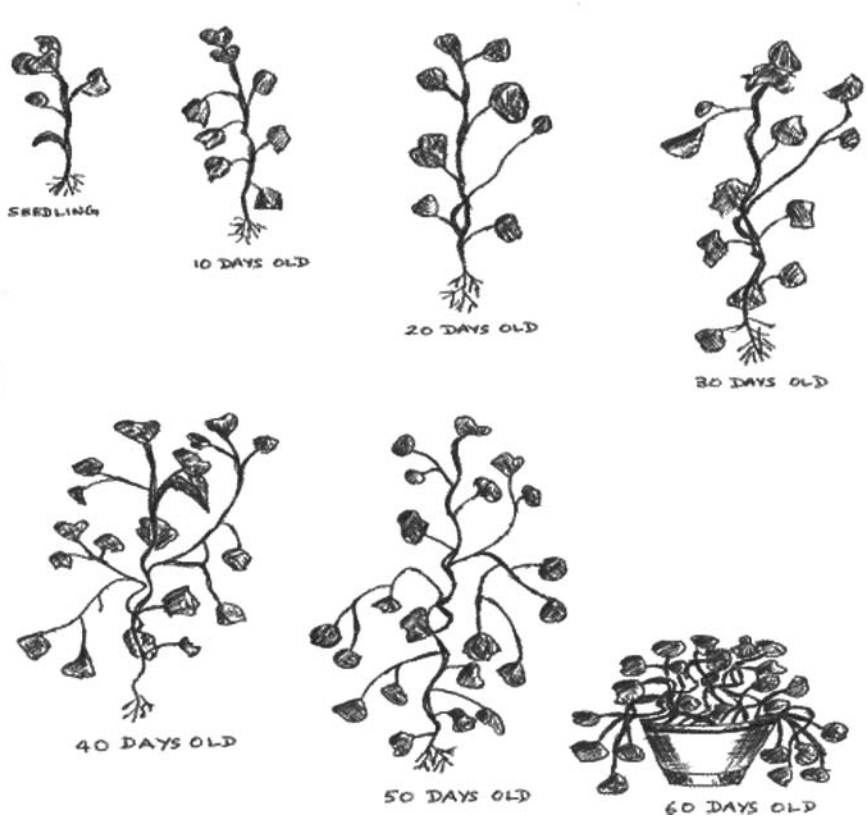

Figure 2.

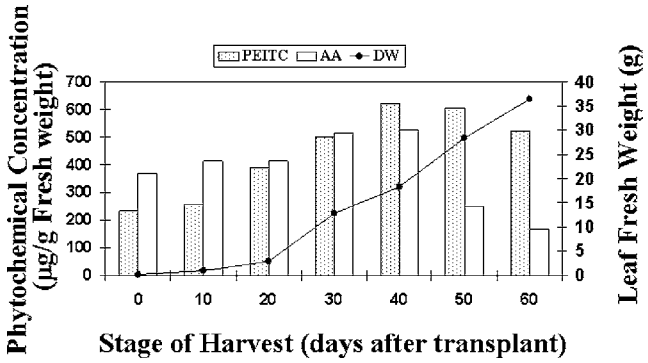

Figure 3.

60 days compared to the plants at 40 days, the PEITC concentrations did not differ in the leaves harvested at 40, 50, and 60 days after transplant. The leaves harvested at 40 days after transplant had higher concentrations of ascorbic acid as well as PEITC.

Ascorbic acid is present in almost all foods of plant origin, providing the major source of the antioxidant vitamin C, an essential micronutrient for humans who are unable to synthesize it due to the absence of L-gulonolactone oxidase. Ascorbic acid has been credited with proven benefits in many human diseases such as atherosclerosis, cancer, and cataracts (*29*−*31*). On the other hand, it has also been suggested that under certain conditions, vitamin C may act as a prooxidant due to the high reactivity of vitamin C with transition metals, including iron (*32*−*36*). Since ascorbic acid is an oxalate precursor, ascorbic acid intake has been reported to cause hyperoxaluria, elevated serum oxalate concentration, and systemic oxalosis in adults with kidney problems (*37*, *38*). Thus, the study of ascorbic acid concentrations at various stages of harvest provides an opportunity to be selective when harvesting produce for specific nutrient content.

The study of glucosinolate concentrations within the plant will determine which tissues and the stage of plant development that contains the highest concentration of these compounds. This will enable the plant tissue to be harvested (selective harvest) for extraction, and purification of these anticancer glucosinolates for use as standards in cell studies as well as for commercial purification and production of dietary supplements for use as nutraceuticals. The significant role of PEITC in chemoprevention has been established in numerous studies, and watercress is evolving as a functional food, and PEITC as a potential nutraceutical (*39*−*43*). This is the first of its kind to study in detail the concentration of PEITC in watercress throughout the vegetative growth period from transplant to prior to the onset of the reproductive phase, providing such information for selective harvest of watercress leaves for potential use as a functional food, or for the production of nutraceuticals.

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