Early Compositional Changes during Postharvest Senescence of Broccoli

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Abstract. Compositional changes during the postharvest senescence of broccoli branchlets held at 20C in the dark were investigated, particularly during the first 24 hours. Major losses of sugars, organic acids, and proteins from floral, middle, and base sections of branchlets were detected during the first 6 hours. Between 12 and 96 hours, free amino acid pools increased (especially the amides glutamine and asparagine) for all sections of branchlets, while ammonia accumulated only in floral sections. Results are discussed in relation to the nature of the processes that set the tissues on the pathways leading to postharvest senescence.

Studies of postharvest senescence in horticultural crops have usually concentrated on mature tissue (e.g., fruit pericarp tissue) in which changes occur relatively slowly. In contrast, organs containing immature tissue that is actively growing at harvest (e.g., asparagus and broccoli) senesce rapidly. Major physiological, biochemical, and molecular changes have been demonstrated within 3 h after harvesting asparagus spears (Davies and King, 1993; Hurst et al., 1993b; King et al., 1990; Lill et al., 1990). Since all crops initially spend a period at ambient temperature after harvest (while the crop is graded and packed), and then frequently during display and sale, an understanding of early changes following harvest is essential for accurate interpretation of subsequent changes, which may include storage at other temperatures.

Fresh broccoli is highly perishable with a storage life of 3 to 4 weeks in air at 0C (Makhlouf et al., 1989; Smith, 1940) and 2 to 3 days in air at 20C (Wang, 1977). As broccoli deteriorates, the head yellows due to degradation of chlorophyll (Wang, 1977). Decay is common on florets during advanced stages of senescence (Aharoni et al., 1985).

Ethylene plays an important role in regulating the yellowing of broccoli florets. Application of exogenous ethylene or propylene hastens yellowing (Aharoni et al., 1985; Tian et al., 1994). Conversely, application of anti-ethylene agents (aminoethoxyvinyl-glycine, CO_2, silver ions, rhizobitoxine, sodium benzoate, benzyladenine, and gibberellic acid) retards yellowing (Aharoni et al., 1985; Wang, 1977). The sensitivity of broccoli florets to exogenous propylene may also increase after harvest (Tian et al., 1993). However, the relationship between ethylene and other physiological changes during postharvest senescence of broccoli has not been characterized.

We have previously described physiological changes occurring early after harvest of broccoli, including decreased CO_2 production and a reduced respiratory quotient (RQ) (King and Morris, 1994). In this paper, we detail compositional changes during the senescence of broccoli held at 20C in the dark for up to 96 h, particularly those changes occurring during the first 24 h. Branchlets were used as they are a useful model system for investigating changes during senescence of broccoli (King and Morris, 1993).

Materials and Methods

Plant material and holding conditions. 'Green Belt' broccoli (*Brassica oleracea* L. var. *italica*) heads were harvested at maturity from a commercial field at dawn, stripped of remaining leaves, and transported to the laboratory within 1.5 h of harvest. Heads were surface-sterilized with 100 ppm sodium hypochlorite (Rushing, 1990).

Three branchlets ($\approx$40 g each; Fig. 1) were excised from each head using a sterile scalpel. The branchlets were obtained from the second layer above the head base. These were considered to represent branchlets of an average size and developmental age on the head. Branchlets were placed in sterilized glass jars (550-ml capacity) and held at 20C in the dark, except for brief periods of light when measurements were being taken. The containers were aerated continuously with humidified, ethylene-free air at a flow rate of $\approx$1.5 liters·h^{-1}. Containers were equilibrated for 1 h before initial measurements.
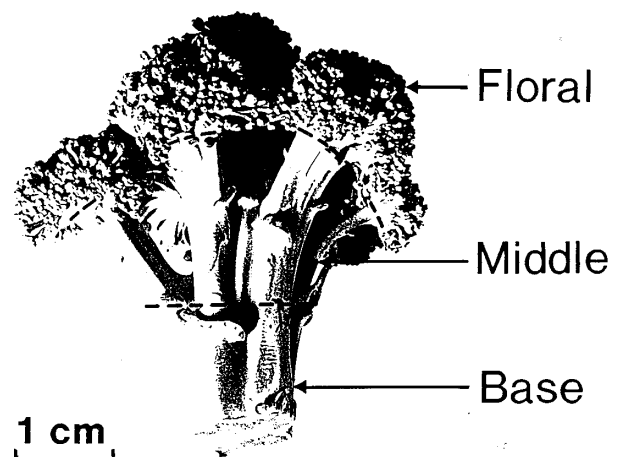

Fig. 1. Sections of broccoli branchlets used for compositional analyses. At each sampling, branchlets were divided into three sections: floral, middle, and base.

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Respiration, ethylene production, and color measurements. Carbon dioxide production and O_2 consumption were measured by infrared gas analysis (PIR-2000; Horiba, Tokyo) and paramagnetic oxygen analysis (Servomex Series 1100; Servomex, Sussex, U.K.) of the outflowing airstream from the glass jars. Ethylene production was measured by injecting 1-ml samples of the outflowing airstream into a gas chromatograph (Loenco, Altadera, Calif.) fitted with a flame-ionization detector (King and Morris, 1993).

Color of florets was measured non–destructively by reflectance using a chromameter (model II; Minolta, Osaka, Japan) with an 8-mm measuring head and D–65 (6504K) illuminant. Reflectance was measured on florets at three equally spaced sites around the circumference of each branchlet. These experiments were replicated five times.

Sample preparation for compositional analyses. A separate batch of branchlets obtained from heads harvested at the same time as those for the physiological studies was used for compositional analyses. At each sampling time, branchlets were cut into three tissue sections (floral, middle, and base; Fig. 1), immediately frozen in liquid N, stored at –20C, and later freeze-dried and pulverized. All assays were performed on duplicate or triplicate samples of the pulverized material. These experiments were replicated three times, with the three branchlets comprising each replicate being obtained from a single head.

Soluble sugar determination. Freeze-dried material (20 mg) was extracted with 0.5 ml 80% (v/v) aqueous methanol at 60C for 15 min (Wade and Morris, 1982). After brief centrifugation ($15,000\times g$, 5 min), 75 to 150 µl of the supernatant were analyzed for soluble sugars using high-performance liquid chromatography (HPLC). Chromatographic conditions were as described by Wade and Morris (1982), using a Waters (Mass.) Si RadialPak modified with tetraethylenepentamine (TEPA). The mobile phase was 80% (v/v) aqueous acetonitrile containing 0.1% TEPA run to waste.

Starch determination . Soluble sugars were removed from 20 mg freeze-dried material by adding 1 ml 80% (v/v) aqueous methanol, vortexing briefly, and centrifuging at $15,000\times g$ for 20 min. The supernatant was discarded and the pellet re-extracted twice more as above.

Starch was solubilized by resuspending the pellet in 0.5 ml 8 M HCl, adding 2 ml dimethylsulfoxide (DMSO), and incubating the samples in a closed flask for 1 h at 60C with gentle shaking. Samples were quickly cooled to room temperature on ice, and 0.5 ml 8 M NaOH were added. The solution was made up to 10 ml with 0.112 M sodium citrate (pH 4.0). Samples were clarified by centrifugation ($15,000\times g$, 5 min). Aliquots (200 µl) of the supernatant were assayed in duplicate for starch using an ultraviolet (UV) starch test kit (Boehringer, Mannheim, Germany) as described in the product literature, except that the final assay volume was 1.16 ml. Recoveries of standard starch ranged from 87% to 94% (n = 3). Data were not corrected for recovery difference.

To confirm that the HCl-DMSO extraction had successfully solubilized the starch present in the tissue sections, the pellets remaining after solubilization were stained with iodine-potassium iodide (O'Brien and McCully, 1981) and examined by microscopy.

Organic acid determination. Organic acids were extracted from 20 mg freeze-dried material with 500 µl 0.1% orthophosphoric acid at 95C for 20 min. The tubes were then centrifuged ($15,000\times g$, 10 min). The pellet was re-extracted as above, and the supernatants combined and made up to 1 ml. A 20-µl aliquot was injected onto a 4-µm radial compression column (C18 Novapak, Waters) running on a HPLC system (Series 6000, Waters). Solvent was 0.1% orthophosphoric acid with a flow rate of 1 ml·min^{-1} and UV detection at 230 nm.

Quantitative protein analysis. Total protein was solubilized by resuspending 20 mg freeze-dried tissue in 1.5 ml 0.1 M NaOH containing 1% (w/v) sodium dodecyl sulfate (SDS) and heating in a boiling water bath for 10 min. Cell debris was pelleted by centrifugation ($15,000\times g$, 10 min) and the pellet was re-extracted as above. Samples (500 µl) of the pooled supernatants were diluted to 1 ml with distilled water. Protein was precipitated by adding 25 µl soluble yeast RNA (5 mg·ml^{-1}; Sigma) and 200 µl 100% (w/v) trichloroacetic acid. Samples were left on ice for 1 h. After centrifugation ($15,000\times g$, 20 min, 4C), the pellet was resuspended in 0.1 M NaOH containing 1% (w/v) SDS. Protein was estimated in triplicate for each sample using the method of Lowry et al. (1951), with bovine serum albumen in 0.1 M NaOH containing 1% (w/v) SDS as standard.

Amino acid and ammonia determinations. Freeze-dried material (20 mg) was extracted with 1 ml 80 ethanol : 20 water (v/v) at room temperature for 15 min while being gently shaken. After centrifugation ($15,000\times g$, 5 min), a 25-µl aliquot was removed and dried under vacuum. This was then derivatized with phenylisothiocyanate to produce phenylisothiocarbamyl amino acids. A 10-liter aliquot was injected onto a column (C18 PicoTag, Waters) held at 38C (PicoTag HPLC System, Waters). The mobile phase consisted of a two-solvent gradient. Buffer A was 150 mM sodium acetate at pH 6.4, and buffer B was 60 acetonitrile : 40 water (v/v). The mobile phase was run with a gradient from 0% to 46% Buffer A over 10 min, then 100% Buffer B for 2 min. Ultraviolet detection was at a wavelength of 254 nm. Ammonia was analyzed by the alkaline hypochlorite/phenol nitroprusside reaction (King et al., 1990).

Results

Respiration, ethylene production, and color changes. Carbon dioxide was initially produced rapidly from branchlets ($\approx$650 mg CO_2/kg per h) upon harvest and storage at 20C in the dark. The rate of CO_2 production then declined during the next 24 h before stabilizing at $\approx$30% of the initial rate (Fig. 2A). The RQ declined in parallel with CO_2 production (Fig. 2A), indicating an accompanying shift toward a more oxidative metabolism soon after harvest.

Ethylene production from branchlets increased markedly at 3 h before declining to near the initial rate by 24 h (Fig 2B). A similar transient increase in wound/stress ethylene production has been found in 'Green Beauty' and 'Dominator' broccoli after harvest, (King and Morris, 1994). Ethylene production varied little between 0.6 to 0.9 nl·g^{-1}·h^{-1} during 24 to 96 h.

Florets commenced yellowing between 48 and 72 h (Fig 2B), as indicated by decreasing hue angle, and yellowed markedly between 72 and 96 h.

Fresh and dry weight distribution, and compositional data. Floral sections (mainly florets and pedicels) comprised $\approx$55% of the initial branchlet fresh weight and contained 13% dry matter. Middle and base sections each comprised $\approx$20% and 25% initial branchlet fresh weight and contained 11% and 8% dry matter, respectively. Florets are actively developing immediately before harvest and contain immature tissue, whereas base sections contain more mature tissue with an extensive pith area. The dry-weight distribution therefore reflects the relative developmental stages and cell sizes of the different sections of the broccoli branchlet.

Branchlets lost less than 5% fresh weight over 96 h at 20C, whereas their dry weight loss was $\approx$18%. All compositional data are therefore expressed per unit fresh weight.
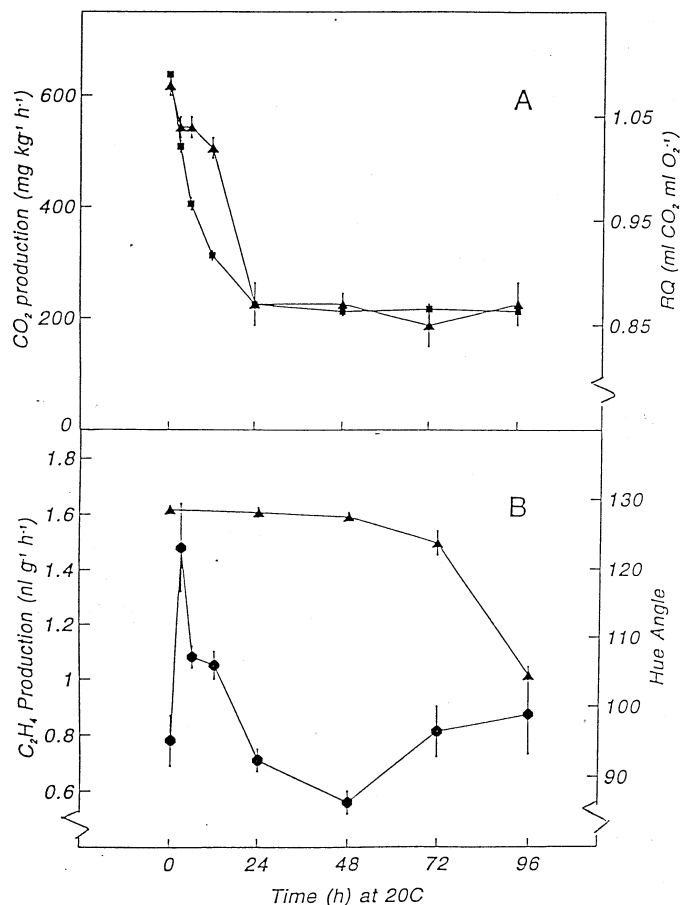
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Fig. 2. Physiological changes in broccoli branchlets held at 20C in the dark. (**A**). CO$_2$ production (square) and RQ (triangle). (**B**). Ethylene production (circle) and hue angle (triangle). Bars are SEs and are contained in the symbols when not shown.
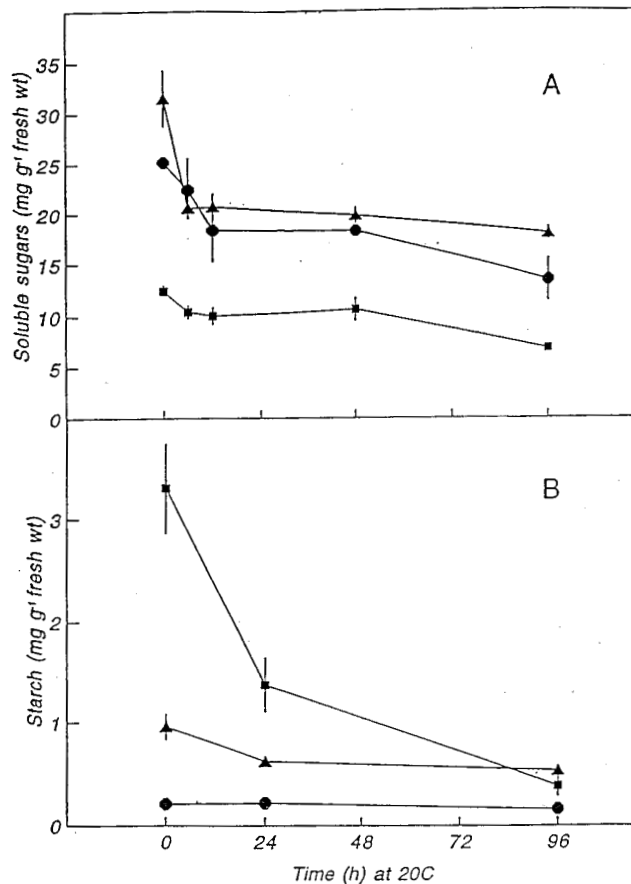

Fig. 3. Changes in soluble sugar (**A**) and starch (**B**) content of floral (square), middle (triangle), and base (circle) sections of broccoli branchlets held at 20C in the dark. Bars are SEs and are contained in the symbols when not shown.

Soluble sugars and starch. Glucose and fructose were the major soluble sugars in all sections of broccoli branchlets initially (Table 1). Sucrose was also present, but at much lower concentrations, and was largely depleted from all sections of branchlets by 24 h (data not presented). The soluble sugar concentration (sum of glucose, fructose, and sucrose) was ≈50% lower in floral sections than in middle and base sections (Table 1, Fig. 3A), both of which contained similar quantities of the individual sugars. The ratio of glucose to fructose did not alter markedly during postharvest senescence. The soluble sugar data presented from 24 h therefore largely reflects concurrent changes in these two sugars.

Soluble sugar content of floral and middle sections declined to an asymptotic level by 6 h (Fig. 3A). Base sections lost soluble sugars during the first 12 h, however levels subsequently remained relatively unchanged (Fig. 3A). These results demonstrate little net loss of soluble sugars from sections of broccoli branchlets from 12 to 96 h. Losses of sucrose accounted for ≈50% of the total sugar lost from floral and base sections during the first 12 h, but only ≈30%

of that lost from middle sections of branchlets (data not presented).

Starch was initially present in significant quantities only in floral and middle sections of branchlets (Fig. 3B). Starch content was highest in floral sections, and declined by ≈50% in both floral and middle sections during 24 h at 20C. Starch was detected only at trace levels in all sections of broccoli branchlets by 96 h. Iodine staining confirmed starch removal during the extraction procedure (data not presented).

Organic acids. Malic acid was the major organic acid present in all sections of broccoli branchlets initially, with citric acid being present at lower levels (Table 2). Both citric and malic acid concentrations changed similarly during postharvest senescence, so combined data for the two are presented (Fig. 4).

The organic acid content of middle and base sections of broccoli branchlets rose at 6 h, and subsequently declined rapidly in all sections by 12 h. Organic acid pools were totally depleted by 24 h in floral sections, 48 h in middle sections, and 72 h in base sections (Fig. 4).

Proteins, amino acids, and ammonia. The total protein content of floral sections declined moderately during the first 12 h, and

Table 1. Major soluble sugars (mg·g^{-1} fresh weight, SE in parentheses) present initially in different sections of broccoli branchlets.

Section	Fructose	Glucose	Sucrose
Floral	4.5 (0.35)	6.9 (0.49)	1.2 (0.64)
Middle	13.3 (0.35)	14.5 (0.67)	3.8 (2.40)
Base	10.4 (0.27)	10.9 (1.27)	4.0 (1.12)

Table 2. Major organic acids (mg·g^{-1} fresh weight, SE in parentheses) present initially in different sections of broccoli branchlets.

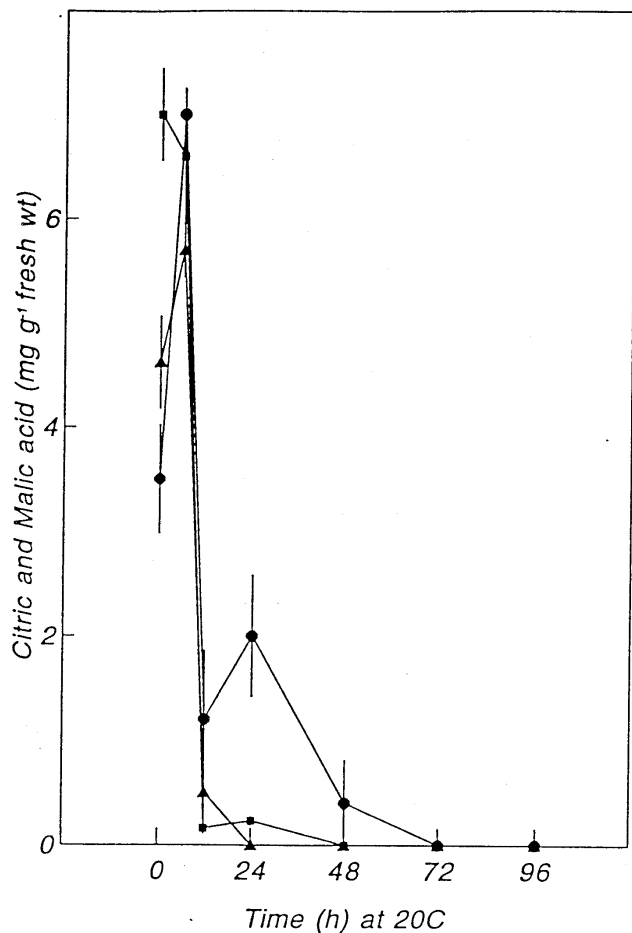| Section	Malic acid	Citric acid
Floral	4.3 (0.84)	2.6 (0.59)
Middle	3.7 (1.21)	1.3 (0.08)
Base	2.6 (0.44)	0.8 (0.13)

Fig. 4. Changes in organic acid content of floral (square), middle (triangle), and base (circle) sections of broccoli branchlets held at 20C in the dark. Bars are SEs and are contained in the symbols when not shown.

Table 3. Changes in free amino acid content (mg·g^{-1} fresh weight, SE in parentheses) in floral sections of broccoli branchlets held at 20C for up to 96 h^z.

Amino acid	Time (h) at 20C		
	0	12	96
Alanine	0.1 (0.03)	0.1 (0.03)	0.1 (0.03)
Arginine	0.2 (0.03)	0.1 (0.01)	0.5 (0.03)
Asparagine	0.1 (0.05)	0.2 (0.04)	1.1 (0.03)
Aspartic acid	0.2 (0.01)	0.2 (0.02)	0.3 (0.01)
Glutamine	0.7 (0.07)	1.4 (0.06)	1.9 (0.10)
Glutamic acid	0.5 (0.09)	0.6 (0.03)	0.4 (0.01)
Histidine	0.1 (0.04)	0.1 (0.02)	0.3 (0.01)
Isoleucine	Trace	0.1 (0.01)	0.2 (0.01)
Leucine	Trace	0.1 (0.01)	0.1 (0.01)
Lysine	Trace	Trace	0.1 (0.01)
Methionine	Trace	Trace	Trace
Phenylalanine	Trace	0.1 (0.01)	0.4 (0.01)
Proline	0.1 (0.01)	0.1 (0.01)	0.1 (0.01)
Serine	0.2 (0.02)	0.2 (0.01)	0.5 (0.01)
Threonine	0.1 (0.01)	0.1 (0.02)	0.1 (0.01)
Tyrosine	Trace	Trace	0.1 (0.01)
Valine	0.1 (0.01)	0.1 (0.01)	0.5 (0.02)
Total	2.5 (0.26)	3.5 (0.11)	6.6 (0.02)

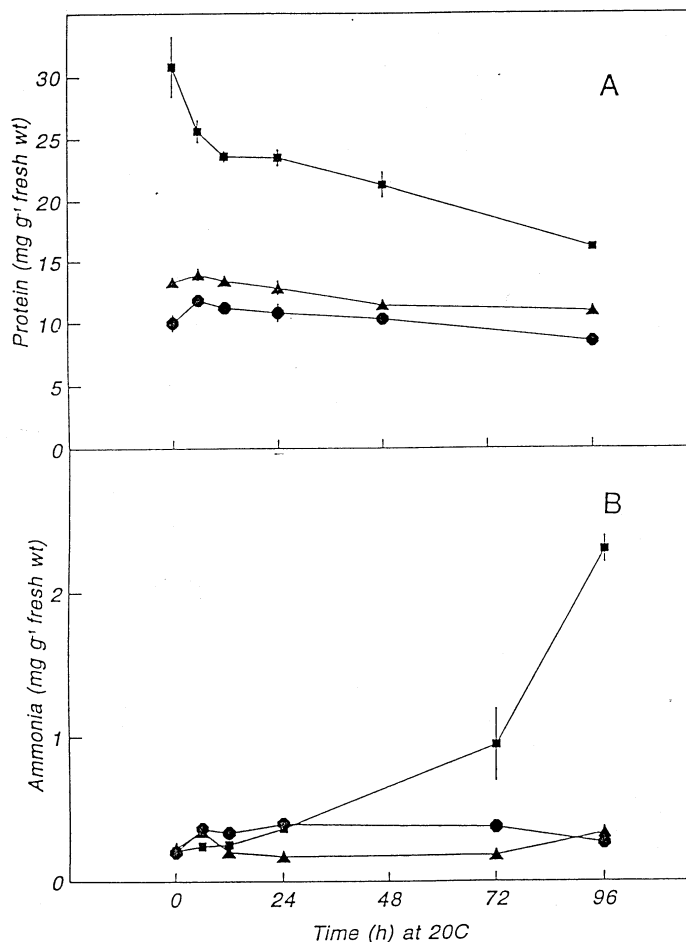^{z}Trace is <0.03 mg·g^{-1} fresh weight.

Fig. 5. Changes in total protein (**A**) and ammonia (**B**) content of floral (square), middle (triangle), and base (circle) sections of broccoli branchlets held at 20C in the dark. Bars are SEs and are contained in the symbols when not shown.

subsequently declined at a slower rate (Fig. 5A). The protein content of middle and base sections declined only slowly between 24 and 96 h.

Amino acid changes in all sections of broccoli branchlets followed similar trends, so only data for floral sections are presented (Table 3). The free amino acid content of floral sections increased during 12 h at 20C, with a doubling of the glutamine pool accounting for most of this increase (Table 3). By 96 h, most free amino acids had increased with asparagine, isoleucine, phenylalanine, and valine all showing greater than 5-fold increases from their initial levels. Deviating results were found only for alanine and glutamic acid (which accumulated to ≈2-fold initial levels only in base sections at 96 h), and arginine (which was depleted from base sections by 96 h; data not shown).

The ammonia content of floral sections increased between 24 and 72 h, and increased further again at 96 h (Fig. 5B). The ammonia content of middle and base sections remained unchanged during senescence.

Discussion

Harvesting and handling broccoli for market imposes a series of stresses on tissue, including wounding, separation from nutrient source, and dehydration. Harvest stresses are particularly severe on organs containing immature tissue that was actively growing at harvest (e.g., broccoli florets). These organs are unable to maintain metabolic homeostasis and senesce rapidly (Huber, 1987).

Carbon dioxide production and RQ of 'Green Belt' broccoli branchlets declined rapidly during 12 h at 20C. Similar early changes in respiratory metabolism have been found in other broccoli cultivars, and may trigger or reflect the onset of postharvest senescence (King and Morris, 1994).

A decline in CO_2 production and RQ was accompanied by losses of metabolites from broccoli branchlets. When the losses of starch, sugars, organic acids, and protein are considered against the respiration requirements (calculated as hexose units) of branchlets over 48 h, there was a greater loss of metabolites than required to account for CO_2 production (theoretical dry weight loss = 10%, reconstructed branchlet metabolite loss = 17%; calculations not presented), especially during the first 12 h (theoretical dry weight loss = 4%, reconstructed branchlet metabolite loss = 12%; calculations not presented). This suggests some use of metabolites for other purposes (e.g., growth or maturation-related processes).

Losses of sugars were greatest in middle and base sections during the first 12 h, and greatly exceeded that required to support CO_2 production from these sections (data not presented). In addition to biosynthetic processes, it is likely that some of these sugars were translocated to floral sections to help maintain the floral sugar pool and support the high respiratory demand of floral sections (King and Morris, 1994). Conversion of starch to glucose and fructose in floral sections during the first 24 h would also contribute to maintaining the floral sugar pools.

The rapid decline in organic acids from all sections of 'Green Belt' broccoli branchlets at 6 to 12 h strongly suggests that these components were oxidized at this time. This does not always occur in other broccoli cultivars (Dostal et al., 1965). Increased levels of organic acids in middle and base sections at 6 h may reflect activation or derepression of phosphoenylpyruvate carboxylase activity to replenish citric acid cycle intermediates as occurs in other stress situations (Tietz and Wild, 1991). Theoretical calculations of RQs for the oxidation of organic acids commonly give ratios around 1.3, the exact value depending on the organic acid being oxidized. As the RQ consistently declined during the first 12 h, sugars, organic acids, and other cellular components were all probably being oxidized by branchlets during this period, with the RQ reflecting a composite value. The rapid loss of protein from floral sections during the first 12 h supports this view. Although no determination of lipid has yet been undertaken, any lipid oxidized would contribute to the low RQ detected.

Use of protein as respiratory substrate when sugars are still present in tissue has been observed. Platenius (1942) provided evidence that proteins were oxidized in addition to sugars during the postharvest senescence of asparagus. Further, Yemm (1937) showed that protein of detached barley leaves held in the dark was used for respiration during the first 12 h when sugars were still present. Some of the sugar pools may be contained in compartments inaccessible to respiratory processes (Saglio and Pradet, 1980). In this case, very rapid depletion of the accessible sugar pools will place tissue under carbohydrate stress. Maintenance of the sugar pools in all sections of broccoli branchlets accompanied by both a low RQ during 12 to 96 h and a total branchlet energy deficit at 96 h (theoretical dry weight loss = 20%, reconstructed branchlet metabolite loss = 17%; calculations not presented), indicates the use of alternative respiratory substrate(s) during the later stages of postharvest senescence.

Changes in the metabolism of nitrogen-containing compounds in addition to protein were also detected in senescing broccoli branchlets. Glutamine and asparagine accumulated to high levels by 96 h, with the two amides collectively accounting for ≈50% of the free amino acids present during late senescence. Amide accu-mulation, together with maintenance of the glutamic acid pool, strongly suggests operation of the glutamine synthetase/aspar-agine synthetase pathway reassimilating ammonia produced by proteolysis in floral sections to fuel respiration under starvation stress conditions (Genix et al., 1990; Hurst et al., 1993a; Peoples and Dalling, 1988; Sieciechowicz et al., 1988).

Accumulation of amides in middle and base sections seems unusual, as large net protein loss occurred only in floral sections. Amides are commonly synthesized as N transport compounds (Rabe, 1990), and their distribution may reflect translocation from the floral tissues to other sections of the branchlet after harvest.

Isoleucine, valine, and phenylalanine accumulated to greater than 5-fold their initial levels by 96 h. Both isoleucine and valine are considered major amino acids of membrane proteins, which are a source of oxidizable substrate for ATP production via β-oxida-tion of their 2-oxo acids (Genix et al., 1990). Isoleucine and valine presumably accumulate because their rate of production exceeds their rate of use by the cells. Breakdown of chloroplast membranes (occurring from 48 h in broccoli florets, M. Vesk, personal communication) is a potential source of both protein and lipid which may be recycled during senescence to provide respiratory substrate. Phenylalanine accumulation is commonly observed in plant tissues placed in starvation conditions (Brouquisse et al., 1992; King et al., 1990). This suggests that phenylalanine is only partially recycled or degraded in these situations.

Ammonia accumulated only in floral sections of branchlets during late senescence. Floral sections have a 4-fold higher respi-ration rate compared with other sections of broccoli branchlets (King and Morris, 1994). Ammonia probably accumulates be-cause of depletion of available carbon skeletons required for its assimilation (Hurst et al., 1993a).

Yellowing of florets 3 to 4 days after harvest is the most striking symptom of the postharvest senescence of broccoli, and can be regulated by ethylene (Aharoni et al., 1985; Tian et al., 1994; Wang, 1977). The onset of yellowing of 'Green Belt' florets was not correlated with major changes in endogenous ethylene produc-tion by entire branchlets during senescence at 20C. Similar results have been found with 'Shogun', 'Dominator', and 'Green Beauty' broccoli branchlets (King and Morris, 1994). However, branchlets contain much vegetative tissue, which may mask significant changes in ethylene production by the reproductive structures of the broccoli florets (Tian et al., 1994). Further, the sensitivity of florets to exogenous propylene increases 24 h after harvest (Tian et al., 1994), and may also influence yellowing.

Yellowing of florets is clearly a relatively late event in the postharvest senescence of broccoli, preceded by major physiologi-cal and compositional changes from very early after harvest. The changes in respiration, carbon metabolism, and nitrogen metabo-lism are consistent with changes described for plants in stress situations. The relationship between these early metabolic changes and increased sensitivity of florets to ethylene and/or changes in ethylene production in floral tissue merits further research.

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