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Quality-related constituents in tea (*Camellia sinensis* (L.) O.
Kuntze) as affected by the form and concentration of nitrogen
and the supply of chloride

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General introduction

1 Tea production

Tea is the second most popular beverage in the world, next to plain water. According to processing procedures, tea currently made in the world can be classified into six main types including black, green, white, yellow, oolong and reprocessed tea. Green tea is called as a non-fermented tea as the first step in its processing is to inactivate enzyme by dry heating or steaming. Most of chlorophyll is preserved and oxidation of catechins is limited. In contrary, black tea is referred to as a full-fermented tea because catechins are oxidized to theaflavins and thearubigins, responsible for the typical color and taste of black tea. Oolong tea is another type called a semi-fermented tea. During manufacturing of oolong tea, young shoots are allowed to withering for a short time (< 8 hrs) before parching to inhibit activity of enzyme. Oxidation of catechins occurs in processing but to a much less extent than those for black tea. The principal types of tea produced and consumed in the world are black and green tea, with small amounts of other types (International Tea Committee, 2003). A summary of world tea production in 2003 and trade in 2002 is shown in Figure 1. Major tea producers are India, China, Sri Lanka, and Kenya, while major consumers are India, China, Turkey, Japan, Russia and United Kingdom (FAO, 2003; International Tea Committee, 2003). The largest annual consumption per capita ($\text{kg head}^{-1} \text{ year}^{-1}$) is Ireland (2.71), followed by Libya (2.65), Kuwait (2.29) and United Kingdom (2.28) (International Tea Committee, 2003). Other countries consuming more than 2 kg per capita per year are Iraq, Qatar and Turkey.

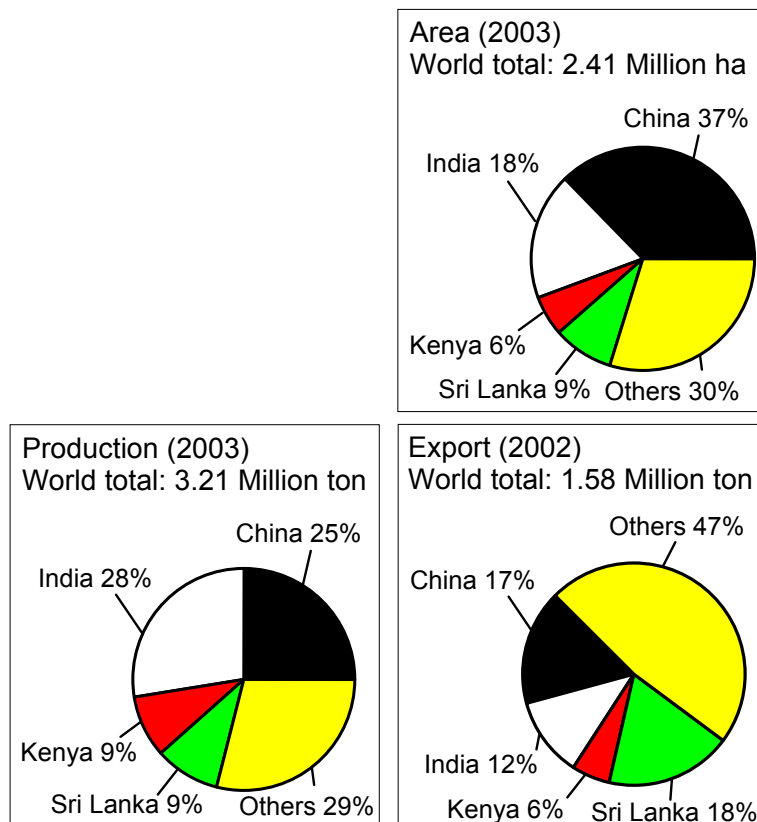


Figure 1 Global tea production in 2003 and export in 2002 (FAO, 2003)

China is the hometown of tea plantation, processing and drinking. There are some records suggesting that tea processing and making into a drinking became a part of daily life in China as early as 2200 years ago (Chen and Shen, 1981). In nowadays, China is the only country producing all of the six types of tea mentioned above. China has the largest area of tea plantation in the world (1207.3 thousand ha) and makes the second largest production (768.1 thousand ton), which consists of green tea, black tea, oolong tea, compressed (reprocessed) tea and others (Figure 2) (Ministry of Agriculture of China, 2003). China is also the second largest tea exporter of the world, exporting 252.3 thousand ton and earning 332 million US\$ in 2002 (Wu, 2003). Most (68%) exported Chinese tea is green tea, making China the number one exporter of this tea type.

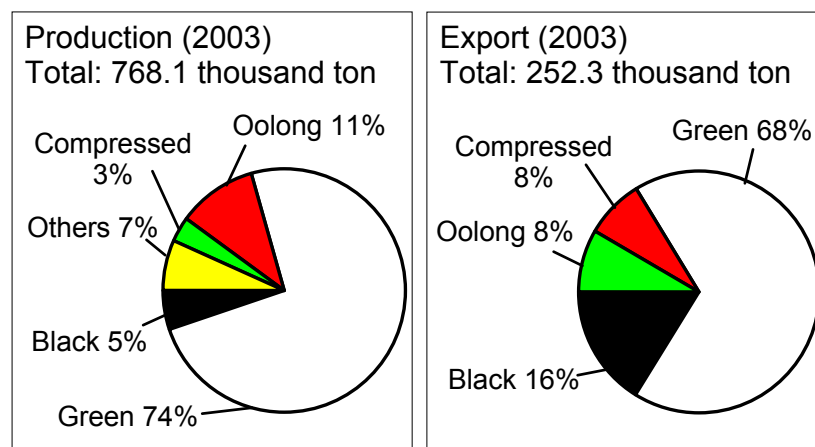


Figure 2 Tea production in 2003 and export in 2002 of China (Ministry of Agriculture of China, 2003; Wu, 2003)

Tea is generally processed from apical buds and the first a few (2-4) tender leaves. Tea shrub, a perennial evergreen plant, is classified in the *Theaceae* family and the *Camellia* genus (*Camellia sinensis*, (L) O. Kuntze). It consists mainly of two varieties, *C. sinensis* cv. *sinensis* and *C. sinensis* cv. *assamica*. In nature, tea plants can attain a height of 20-30 m but are often pruned to shrubs in plantations. Tea plants were probably originated in southeast China (Tea Research Institute, 1985) and currently are grown in countries located in a wide range of latitudes in the world, from 45 °N (Russia) to 30 °S (South Africa), and longitudes from 150 °E (New Guinea) to 60 °W (Argentina) (Huang, 1989). In tropical countries, young shoots are harvested all year around. In temperate countries, harvesting is seasonal.

2 Chemical compositions related to tea flavor

2.1 The flavor of tea

Theoretically, fresh young shoots of tea plants can be processed into any types of tea. However, in practice young shoots from a specific cultivar may only be suited for processing into one of the six types of tea depending on physical, chemical and biological characteristics of the young shoots. For instance, tea cultivars suitable for black tea (generally belonging to *C. sinensis* cv. *assamica*) are not considered as a good

choice for green tea and oolong tea because of high polyphenol contents. The latter two types of tea are usually produced from *C. sinensis* cv. *sinensis*. Good quality oolong tea requires cultivars with thick leaves, medium to dark leaf color, and medium polyphenol contents (Lu, 1987).

Quality of tea is determined by its appearance, color, and most importantly by its flavor. The flavor of tea comprises taste and aroma. The taste is characterized by astringency, bitterness, mellowness and slight sweetness (Hara et al., 1995). The aroma is often described as fresh, fragrant, flowery or dull and grassy. For long time, it has been well established that the flavor of tea is principally determined by chemical components it contains, such as volatile compounds contributing to the property of aroma and nonvolatile compounds to the taste (Bondarovich et al., 1967; Hara et al., 1995; Kubota and Hara, 1976; Millin et al., 1969; Nakagawa, 1975; Nakagawa et al., 1981). These compounds are either direct metabolites accumulated in young shoots during growth or products of various reactions using these metabolites as substrate during processing. Thus the flavor of made tea is largely affected by abundance of chemical constituents and their relative composition in young shoots. Table 1 presents the most important constituents related to tea flavor and their typical concentrations in fresh young tea shoots.

Table 1 Chemical compounds in young tea shoots and their typical concentrations (dry weight base)

Compound	Concentration in young shoots
Polyphenols	20-40%
Flavan-3-ols (catechins)	10-30%
Flavonols and glycosides	~1%
Flavones	1-2%
Anthocyanins	~1%
Phenolic acids	2-3%
Free amino acids	1-5%
Theanine	0.5-2.5%
Alkaloids	2-5%
Caffeine	2-5%
Theobromine	~0.2%
Theophylline	~0.05%
Carbohydrates	20-30%
Soluble reduced sugars	0.8-4%
Polysaccharide	20-26%
Protein	14-17%
Lipids	4-9%
Pigments (chlorophyll, carotenoids, xanthophyll)	~1%
Minerals (P, K, Ca, Mg, Al, Mn, ...)	~6%
Volatile compounds	0.01-0.02%

2.2 Chemical compositions in young tea shoots and their relation with tea flavor

2.2.1 Amino acids

Concentrations of total free amino acid (including amides) in tea range from 1 to 5%. The most abundant components are theanine (Thea), glutamine (Gln), glutamic acid (Glu), and arginine (Arg) (Figure 3). L-theanine (N⁵-ethyl-glutamine), the predominant free amino acid in tea, is so far found only in a few other species of *Camellia* at low concentrations and a mushroom *Xerocomus badius* (Casimir et al., 1960; Tsushida and Takeo, 1984). Thea is not incorporated into protein and exists in free state. Its concentration accounts for up to 70% of total free amino acid. In addition to L-theanine, there is D-theanine (up to 3% of the total concentration of theanine) in tea (Ekborg-Ott et al., 1997).

Each free amino acid (or amide) has its own taste as one of or in combination of sweet, salty, sour, bitter and umami (Table 2). The Japanese word 'umami' means 'delicious' and represents the characteristic sensory property of monosodium glutamate. Free amino acids infused in a green tea brew have been recognized as the principal contributor to the taste of mellowness (Chen et al., 1985; Mukai et al., 1992; Wang et al., 1988; Nakagawa et al., 1977; 1981). Some volatile compounds found in made tea are derived from free amino acids. Amino acids may be decarboxylated and deaminated with resultant products of aldehydes (Sanderson and Graham, 1973). In the presence of flavonoids, amino acids can undergo Strecker degradation to form aldehydes, alcohols and carboxylic acids (Co and Sanderson, 1970; Saijo and Takeo, 1970 a; b). Under heated condition during manufacturing process, free amino acids especially L-arginine and L-theanine may react with sugars to form furan, pyrazine and pyrrole, which contribute to the roast aroma of green tea (Hara, 1981; Kosuge et al. 1981; Hara and Kubota, 1974; 1982a; b; 1983a; Kawakami and Yamanishi, 1983).

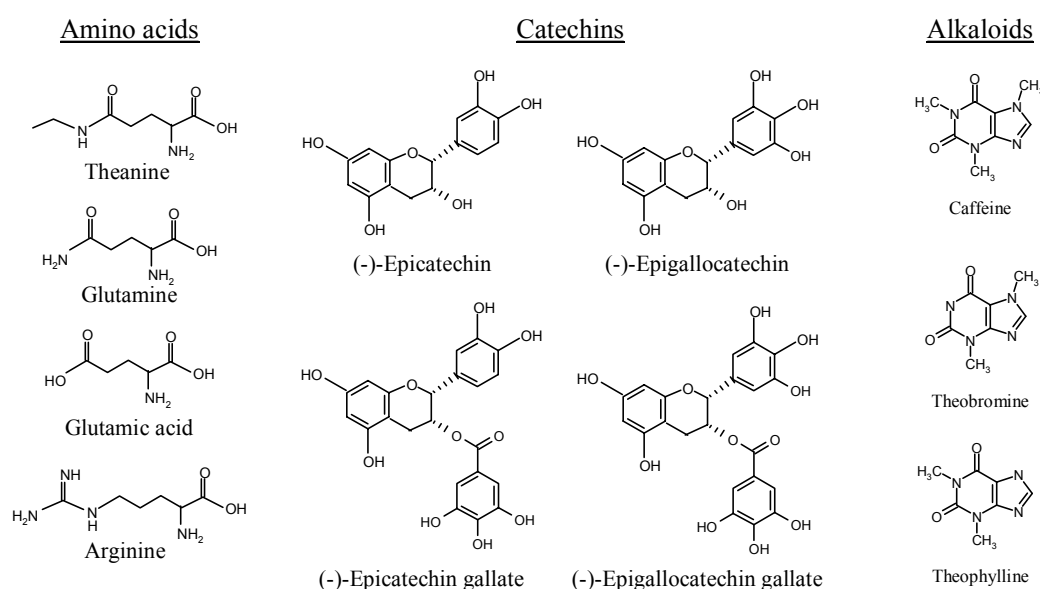


Figure 3 Chemical structures of major amino acids, catechins and alkaloids in tea

Table 2 Taste of L-amino acids

Taste type	Amino acid	Stimulus threshold (mg/100 mL) ^{a)}	Differential threshold ^{b)}	Sweet	Salty	Sour	Bitter	Umami
Sweet	Gly	110	10%	+++ ^{c)}				
	Hypro	50	-	+++			++	
	Ala	60	10%	+++				+
	Thr	260	7%	+++				
	Pro	300	50%	+++			+++	
	Ser	150	15%	+++				+
	Cit	500	20%	++			++	
	Lys/HCl	50	20%	++			++	+
	Gln	250	30%	+				+
		Theanine ^{d)}						
Bitter	Phe	150	20%				+++	
	Trp	90	10%				+++	
	Arg	10	20%				+++	
	Arg/HCl	30	-	+			+++	
	Ile	90	15%				+++	
	Val	150	30%	+			+++	
	Leu	380	10%				+++	
	Met	30	15%				+++	+
	Orn	20	20%	+			++	
	His	20	50%				++	
Sour	His/HCl	5	-		+	+++	+	
	Asp	3	30%			+++		+
	Glu	5	20%			+++		+
	Asn	100	30%			++	+	
Umami	Glu/Na	30	-	+	+			+++
	Asp/Na	100	20%		++			++
	Theanine ^{d)}	150						

^{a)} Stimulus threshold = the minimum concentration at which taste can be perceived.

^{b)} Differential threshold = the minimum difference in concentration that can be perceived.

^{c)} +++: Intensive taste; ++: Strong taste; +: Weak taste

^{d)} Theanine tastes umami and sweet (Hara et al., 1995), but the data on its taste intensity are not available.

Source: http://www.ajinomoto.co.jp/amino/e_aminoscience/bc/b-7.html

2.2.2 Polyphenols

Flavonoids, which were previously called tea tannins (Bokuchava and Skobeleva, 1969) and also are referred to as tea polyphenols (Harboy and Balentine, 1997), are main phenolic compounds comprising 20-40% of dry matter in young shoots of tea plants. The characteristics of made tea, including color, taste and aroma are directly or indirectly associated with these phenolic compounds (Wang et al., 2000). The predominant flavonoids in tea are catechins (flavan-3-ol) and their gallates: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (Figure 3), generally accounting for up to 30% of dry weight (Harbowy and Balentine, 1997). EGCG is the most abundant catechin, followed by EGC, ECG, and EC. The flavanol class contains some digallates such as

flavanol-3,5-digallates (Coxon et al., 1972). Other flavonoids include anthocyanins, flavonols (quercetin, kaempferol, myricetin) and their glycosides (Engelhardt et al., 1992; 1993; Finger et al., 1991a; b; 1992; Lakenbrink et al., 2000a; McDowell et al., 1990; Rio et al., 2004; Scharbert et al., 2004), flavones (Chaboud et al., 1986; Engelhardt et al., 1993; Kiehe and Engelhardt, 1996), proanthocyanidins (Hashimoto et al., 1989; Kiehne et al., 1997; Lakenbrink et al., 1999; Nonaka et al., 1983; 1984), and phenolic acids (gallic acid, chlorogenic acid and theogallin) (Bailey et al., 1990; Cartwright and Roberts, 1954; Kuhr and Engelhardt, 1991; Roberts and Myers, 1958; Shao et al., 1995; Ullah and Jain, 1980).

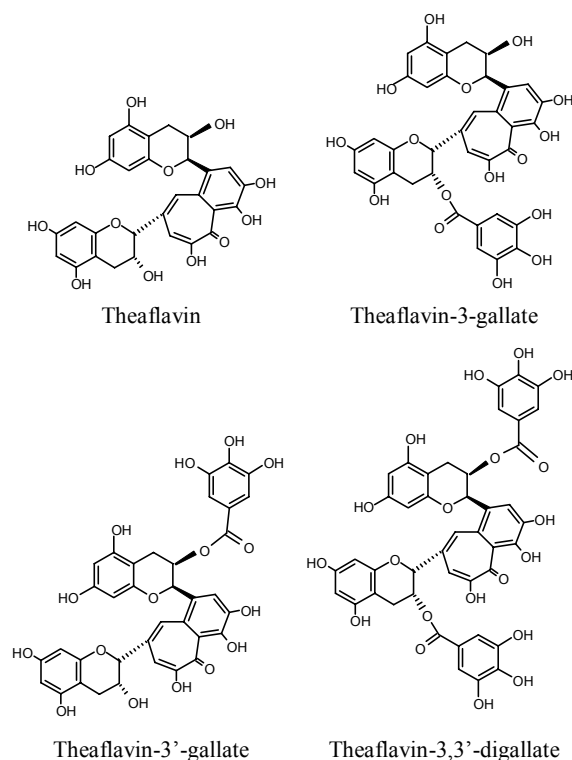


Figure 4 Chemical structures of major theaflavins in black tea

In black tea production, catechins are oxidized, catalyzed by polyphenol oxidase, to form pigments theaflavins (TFs) and thearubigins (TRs), which give typical color and flavor of black tea (Coxon et al., 1970a; Roberts et al., 1957; 1958a; 1958b; Takino et al., 1964). The structure of the main theaflavins has been determined in 1960s (Takino et al., 1965; 1967; Brown et al., 1966; Bryce et al., 1970). There are four principal TFs pigments in black tea, referred to as theaflavin (TF), theaflavin-3-gallates (TF3G), theaflavin-3'-gallates (TF3'G) and theaflavin-3,3'-digallates (TFDG) (Figure 4) (Collier et al., 1973). Traces of epitheflavic acids, theaflavic acids, epitheflavic acid-3'-gallate, isotheaflavin, and neotheaflavin are also present in black tea (Bryce et al., 1972; Coxon et al., 1970b; c) and new compounds belonging to this group are continuously identified (Sang et al., 2002; Tanaka et al., 2002; Wan et al., 1997; Zhu et al., 2000). All these theaflavins and related compounds possess a similar unique benzotropolone ring, formed by co-oxidation of selected pairs (di- and trihydroxylated) of catechins or with gallic acid (Roberts, 1958a; b; Takino et al., 1964; 1965; 1967). This characteristic structure produces yellowish and bright reddish colors and provides a bright and yellowish appearance to the beverage. The content

of total theaflavins in black tea is usually less than 2.5% (Balentine et al., 1997; Graham, 1992; Owuor and Obanda, 1995; Steinhaus and Engelhardt, 1989). Another group of red-brown or dark-brown pigments is called thearubigins (TRs). The structure of TRs is poorly understood (Bailey et al., 1991; Brown et al., 1969a; b; Cattell and Nursten, 1976; 1977; Degenhardt et al., 2000; Menet et al., 2004; Millin et al., 1969; Ozawa et al., 1996; Roberts, 1958a; b; Roberts et al., 1957; Robertson and Bendal, 1983; Wedzicha and Donovan, 1989; Wedzicha et al., 1988). TRs are heterogeneous polymers and considered as products of further oxidation of theaflavins (Roberts, 1958; 1962; Subramanian et al., 1999), products of polymeric proanthocyanidins (Brown et al., 1969a; b), oxidation products of catechins and catechin-catechin interactions other than formation of benzotropolone (Robertson, 1983; 1992) or even products of interactions of catechin quinones with other macromolecules such as proteins, carbohydrates and nuclei acids (Robertson, 1992).

Catechins and their oxidation derivatives are the principal contributor to astringent and bitter tastes of tea beverages (Millin et al., 1969; Nakagawa, 1970; Scharbert et al., 2004; Zhang et al., 1992). The taste thresholds of individual catechins range from 18.1 to 51.0 mg/100 mL (Nakagawa, 1970) or from 190 to 930 $\mu\text{mol L}^{-1}$ (Scharbert et al., 2004). Galloyl esters show lower taste thresholds than nongalloylated catechins. Catechins are responsible for the astringent taste and strength of green tea infusion. During green tea manufacture, most catechins and other polyphenols are preserved owing to inactivation of enzyme by dry heating or steaming at the initial step. Green tea quality positively correlates with concentration of polyphenols (Chen et al., 1985; Lu et al., 1994; Wang et al., 1988). However, high concentration of polyphenols or catechins, which makes the infusion strongly bitter, astringent and less fresh, is not necessarily required for quality green tea. Quality green tea is characterized by high contents of free amino acids with appropriate concentrations of catechins and caffeine (Chen et al., 1985; Wang et al., 1988).

Pure TFs have a taste of very fierce astringency (Millin et al., 1969), inducing a mouth-drying and rough-astringent oral sensation (Scharbert et al. 2004). The taste thresholds of TFs (between 13 and 26 $\mu\text{mol L}^{-1}$) are much lower than their precursor catechins. TFDG possesses the strongest astringency among the major TFs (Owuor and Obanda, 1995; Scharbert et al., 2004). Thearubigins are responsible for body, richness and fullness of the tea brew (Roberts, 1962). Previous experiments have shown significant contribution of TFs and TRs to the quality of black tea (Biswas et al., 1973; Hazarika et al., 1984; McDowell et al., 1995; Roberts, 1958ab, 1962). Consequently as precursor substances for TFs and TRs, concentrations of catechins in fresh young shoots positively correlate with black tea quality (Biswas et al., 1973; Hilton and Palmer-Jones, 1973; Obanda et al., 1997).

Other polyphenols, such as flavonols and their glycosides, gallic acid, chlorogenic acid, though not influencing the color of tea, have been link to the taste of astringency (Bailey et al., 1990; Haslam, 1989; Scharbert et al., 2004). Some flavon-3-ol glycosides, which usually do not undergo significant changes during processing, induce a silky, mouth-drying and mouth-coating sensation with extremely low taste thresholds spanning from 0.001 to 19.8 $\mu\text{mol L}^{-1}$ (Scharbert et al., 2004). The sensory

activity of these flavon-3-ol glycosides is influenced by the structure of aglycon, sugar moiety and sequence of the individual monosaccharides in glycosidic chain.

2.2.3 Caffeine

Caffeine (1,3,7-trimethylxanthine) is one of the major alkaloids occurring abundantly in tea (Figure 3). Its content in young tea shoots ranges from 2 to 5% (Table 1). Other important alkaloids in tea are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethyl-xanthine) (Figure 3). Caffeine is synthesized from xanthosine via a xanthosine → 7-methyl-xanthosine → 7-methylxanthine → theobromine → caffeine pathway (Ashihara and Crozier, 2001). Pure caffeine tastes unpleasantly bitter (threshold is approximately 3 mg L⁻¹ in water) but its taste is modified and is less bitter when present with catechins and theaflavins (Millin et al., 1969). This is because in tea brew, a portion of caffeine can form complex with catechins, theaflavins and thearubigins, and alleviates astringency of the latter compounds. The quality of tea positively correlates with concentration of caffeine which contributes to briskness of black tea infusion (Lu et al., 1994; Obanda et al., 1997). In cooled black tea brew, cream is formed as a complex of caffeine, catechins, theaflavin gallates, polysaccharides, protein and some other compounds (Liang et al., 2002; Roberts, 1963; Robert and Smith, 1961). The creaming property is an important indicator of black tea quality.

2.2.4 Chlorophyll and carotenoids

Chlorophyll and carotenoids are the major pigments in fresh tea shoots. Total chlorophyll (a and b) varies from 1 to 7 mg g⁻¹ in tea shoots (Mahanta and Hazarika, 1985). Six species of chlorophyll (chlorophyll a and b, their degradation products pheophytin a, chlorophyllide, and pheophorbide species) have been identified in fresh leaves (Figure 5) (Suzuki and Shioi, 2003). Chlorophyll epimers and pyropheophytins are not found in young shoots. Most of chlorophyll is preserved during manufacturing of green tea, making this tea in green color. However, some chlorophyll is transformed to pheophytin and epimers due to heating treatment during processing (Suzuki and Shioi, 2003). In black tea, chlorophyll degrades and transforms to either pheophytin or first to chlorophyllide, catalyzed by chlorophyllase (Kuroki et al., 1981), and finally to pheophorbide. Pheophytin is a darker pigment with 10% higher specific absorbance at 665 nm compared to pheophorbide (Mahanta and Hazarika, 1985). Since pheophytin is black and pheophorbide is brown in color, their relative proportion determines the blackness, an important criterion in evaluation of made black tea (Wickremasinghe and Perera, 1966). Nevertheless, some chlorophyll remains unchanged (Mahanta and Hazarika, 1985; Obanda and Owuor, 1995) and the residual chlorophyll has been linked to dull infusion and grassy taste of black tea (van Lelyveld et al., 1990; van Lelyveld and Smith, 1989). However, some studies suggested that the influence of chlorophyll and breakdown products on black tea quality might be dependent on levels of other chemical compounds, such as thearubigins and theaflavins (Mahanta and Hazarika, 1985; Obanda and Owuor, 1995). Theaflavins at high concentrations can alleviate or mask the negative influence

of chlorophyll on the appearance of black tea infusions (Obanda and Owuor, 1995). Oolong tea, a semi-oxidation tea, contains pheophytin b and high concentration of pyropheophytin a but low concentrations of chlorophyll a and b (Suzuki and Shioi, 2003).

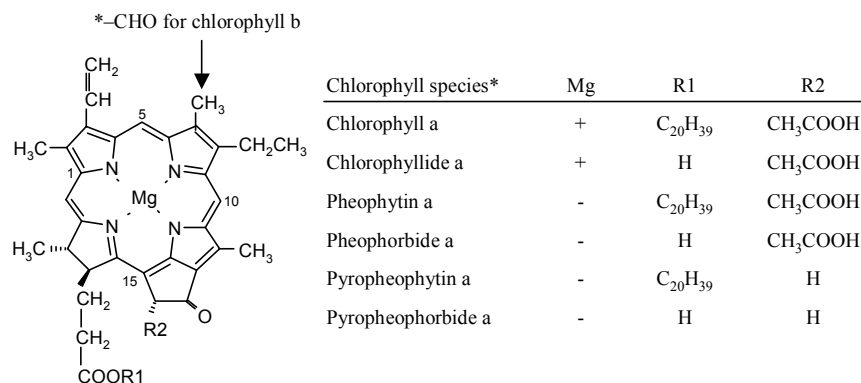


Figure 5 Chemical structures of major components of chlorophyll in tea (Suzuki and Shioi, 2003)

Concentrations of carotenoids in tea shoots range from 0.36 to 0.73 mg g⁻¹ dry weight (Ravichandran, 2002). A total of 14 species of carotenoids is identified in fresh shoots (Suzuki and Shioi, 2003). β -carotene, lutein, violaxanthin and neoxanthin are the predominant components (Hazarika and Mahanta, 1983; Ravichandran, 2002; Sanderson et al, 1971; Suzuki and Shioi, 2003). Carotenoids serve as substrate for many volatile compounds found in made tea (Sanderson and Graham, 1973). During processing, carotenoids degrade via oxidation during withering and fermentation or paralytic reaction during firing (Sanderson and Graham, 1973). In green tea a change of structure, conversion of violaxanthin to auroxanthin occurs (Suzuki and Shioi, 2003). Volatile compounds of ionine series are formed as primary oxidation products of carotenoids (Etoh et al., 1980; Reynold et al., 1974; Sanderson et al., 1971; Sanderson and Graham, 1973; Tirimanna and Wickremasinghe, 1965). These compounds smell sweet floral aroma and are believed to have a major effect on the aroma of tea (Sanderson and Graham, 1973).

2.2.5 Lipids

In young tea shoots, lipids make up 4 to 9% of the dry matter (Mahanta et al., 1985). Glycolipids are the most abundant constituent accounting for more than half of the total lipids content, followed by neutral lipids (ca. 35%) and phospholipids (ca. 15%) (Bhuyan et al., 1991; Bhuyan and Mahanta, 1989; Ravichandran and Parthiban, 2000). Linolenic, linoleic and palmitic acids are major fatty acids (Mahanta et al., 1985; Muritu et al., 1988; Owuor, 1990). Linolenic acid is rich in glycolipids while a high proportion of linoleic and palmitic acids occurs in phospholipids (Bhuyan et al., 1991; Ravichandran and Parthiban, 2000). Neutral lipids contain high contents of lauric, myristic, palmitic, stearic, oleic and linoleic acids. Lipids content increases from bud to third leaf and is low in stem (Mahanta et al., 1985; Owuor, 1990). This variation is related to maturity of chloroplast (Bhuyan and Mahanta, 1989).

Compounds such as (Z)-3-hexenol, its esters and (E)-2-hexenal, which principally contribute to the fresh and greenish odor of green tea, are oxidation products of free fatty acids formed in manufacturing (Hatanaka et al., 1976; Hatanaka and Harada, 1973; Saijo and Takeo, 1972; Selvendran et al., 1978; Wright and Fishwick, 1979). Four enzymes, including lipoxygenase and hydroperoxide lyase [hydroperoxide isomerase] (bound to the thylakoid membrane of chloroplasts of green leaves), catalyze this pathway (Hatanaka, 1993; Hatanaka et al., 1979a; b; 1982a; b; Matsui et al., 1991). Lipoxygenase adds oxygen stereoselectively to the unsaturated fatty acids to produce 13-(S)-hydroperoxides. Hydroperoxide lyase cleaves the bond between C-12 and C-13 of these hydroperoxides to form C₆-aldehydes. There are three lipoxygenase isoenzymes and the enzyme activity increases with the maturation of tea shoots (Mahanata et al., 1995).

2.2.6 Carbohydrates

Free sugars in young tea shoots are mainly glucose, fructose, sucrose, raffinose and stachyose. Starch is low in young shoots but occurs in high concentration serving as the main reserve polysaccharide in roots. Pectin substances contain galactose, arabinose, galacturonic acid, rhamnose and ribose. With heating during the green tea processing, sugars can react with free amino acids to produce compounds with roast aroma (Hara, 1981; Hara and Kubota, 1982a; 1982b; 1983a; 1983b; Kawakami and Yamanishi, 1983; Kosuge et al. 1981). The slight sweetness of tea brew may be accounted for partly by the existence of sugars.

2.2.7 Volatile compounds

Aroma property of teas is intensively investigated (for reviews, Bokuchava and Skobeleva, 1986; Hara et al., 1995; Howard, 1978; Robinson and Owuor, 1992; Yamanishi, 1981). Compounds contributing to tea aroma are primary products present in fresh leaves, which are biosynthesized by plants and do not undergo changes, or are secondary products formed during manufacturing process from substrates (such as lipids, amino acids, carotenoids, etc.) through enzymatic and non-enzymatic reactions. More than 600 volatile compounds concerning with tea aroma have been identified (Hara et al., 1995) and the number is still increasing as new compounds are continuously reported (e.g. Kumazawa and Masuda, 1999; 2002). These compounds are often classified into groups including hydrocarbons, alcohols, ketones, aldehydes, lactones, phenolic compounds, acids, esters, nitrogenous and sulphur compounds and miscellaneous oxygenated compounds (Ito et al., 2002; Mick and Schreier, 1984; Shimoda et al., 1995a; b).

Alcoholic compounds such as geraniol, linalool and its oxides are the main sources of floral flavor of tea. In fresh tea shoots, only a limited proportion of these alcoholic compounds exists in the free state whilst most of them is present as glycosides (Takeo, 1981; Wang and You, 1996; Yano et al., 1990). These glycosides have been isolated and identified, including β -D-glucosides, β -primeverosides and β -vicianosides (Guo et al., 1993, 1994; Kobayashi et al., 1994; Moon et al., 1994, 1996; Nishikitani et al., 1996, 1999). The aglycone moieties can be monoterpene alcohols

(geraniol, linalool, and linalool oxides), aromatic alcohols (benzyl alcohol, 2-phenylthanol), phenol (methyl salicylate) and aliphatic alcohol [(Z)-3-hexenol]. During manufacturing, aglycone moieties are released by endogenous glycosidase contributing to formation of aroma of made teas (Ijima et al., 1998; Ogawa et al., 1995; Ogawa et al., 1997).

The aroma property of a specific tea is determined by manufacturing process, tea variety, ontogeny age of the fresh young shoots, climatic conditions, and cultivation management. The three major types of tea, i.e. green tea, black tea and oolong tea have different aromatic profile. Green tea has flavor of freshness, flower and roast. The fresh and greenish odors are typical characteristics of green tea and are contributed mainly from (Z)-3-hexenol, its esters and (E)-2-hexenal (Kawakami and Yamanishi, 1981; Takei et al., 1976). The distinct roast aroma of pan-firing green tea (such as most Chinese green tea) is generated by pyrroles, pyrazines and furans (Hara and Kubota, 1974; 1983b; Kawakami and Yamanishi, 1983; Kumazawa and Masuda, 1999; 2002). On the other hand, black tea often has large contents of linalool and its oxides, geraniol, methylsalicylate, (E)-2-hexenal, (Z)-3-hexenol, and (E)-2-hexenyl formate (Takeo, 1983). Oolong tea has great proportion of (Z)-jasmone, jasmine lactone, methyl jasmonate and indole but comparatively little hexenol and its esters. Linalool and geraniol are considered as the principal compounds contributing to flowery odor of all three types of tea.

3 Health benefits of tea consumption

Health benefits of tea consumption have been intensively investigated and a fast growing body of scientific research in the last decade indicates a role for tea in the promotion of health and prevention of disease (Blumberg, 2003). The antioxidant, anticarcinogenic, antimutagenic and anti-microbial functions of tea have been repeatedly confirmed *in vitro*, *ex vivo* and in animal models and are principally attributed to the rich contents of catechins or their oxidation derivatives theaflavins and thearubigins (Higdon and Frei, 2003; Lambert and Yang, 2003; McKay and Blumberg, 2002; Rice-Evans et al., 1997; Trevisanato and Kim, 2000; Yang and Landau, 2000). As a unique free amino acid in tea, Thea possesses human health promoting features such as improving immunity (Kamath et al., 2003), producing a relaxation effect, lowering blood pressure and improving memory and learning abilities (Juneja et al., 1999). Some recent studies present evidences that caffeine, a major constituent in tea, is able to inhibit carcinogenesis in UVB-exposed mice (Lu et al., 2002). However, it is also noted that epidemiological evidence regarding the effects of tea consumption on cancer and cardiovascular disease risk is conflicting (Higdon and Frei, 2003) and the absorption and bioavailability of plant phenols to humans have been inadequately studied (Hollman, 2001).

4 Mineral nutrition of tea plant and its relation with the formation of chemical components related to tea flavor

The chemical constituents, which greatly affect the flavor of made tea, are derived from metabolic pathways of biosynthesis during the growth of young shoots.

For example, free amino acids are products of N metabolism while flavonoids are synthesized from phenylalanine through phenylpropanoid pathway (Winkel-Shirley, 2001). Therefore, biosynthesis and accumulation of these quality-related metabolites in young shoots are largely affected by nutrient supply.

4.1 Nitrogen

The presence of the unique amide theanine (Thea) in tea is a specific characteristic of N metabolism for this plant species. Thea is synthesized in root from glutamic acid and ethylamine catalyzed by L-glutamate ethylamine ligase (Konishi and Kassai, 1968; Sasaoka et al., 1965). Ethylamine is formed from decarboxylation of alanine (Takeo, 1974). Thea is translocated in xylem to young shoots where it either accumulates or breakdowns into glutamic acid and ethylamine by hydrolase enzyme (Tsushida and Takeo, 1985). The ethylamine is further oxidized to acetaldehyde and NH_2 is released (Tsushida, 1987). The exact physiological functions of Thea in tea plants have not been clearly understood. It is suggested that Thea possibly functions as an organic N form for transport from root to shoot and for storage (Feldheim et al., 1986). The N-ethyl carbon of Thea is significantly incorporated into the phloroglucinol ring (A ring) of catechins, which is controlled by light intensity (Kito et al., 1968), indicating that Thea may play a role in formation of catechins in young shoots.

Some experiments compared the effect of different N forms (NH_4^+ vs NO_3^-) and showed tea plants grow better with NH_4^+ than with NO_3^- in sand culture (Ishigaki, 1974; 1978) and under soil conditions (Hoshina, 1985). Short-term (24 h) nutrient solution experiment using ^{15}N also demonstrated larger absorption of NH_4^+ over NO_3^- when both sources are supplied at similar concentrations (Morita et al., 1998). Total N content in leaves is increased by application of NH_4^+ together with a nitrification inhibitor as compared to application of NO_3^- (Ruan et al., 2000). Field experiment comparing the effects of ammonium sulphate and calcium ammonium nitrate showed the latter N fertilizer produced similar or less yield increase (per kg N), leading to the conclusion that 'the yield data do not eliminate conclusively the possibility that nitrogen as nitrate is less effective for tea than nitrogen as ammonium' (Willson, 1975a). However, other factors might have played roles in determining the effect of N fertilizers. NO_3^- compared to NH_4^+ is readily leached in humid tea growing areas and the calcium concomitantly applied with NO_3^- could be directly toxic to tea plants or induce K deficiency (Willson, 1975b; c). The effect of different N forms is variable depending on the pH; NH_4^+ -fed plants had better growth at higher pH in the range from 3 to 6.5 (Ishigaki, 1974), which is however less convincing since the pH was not adjusted timely. The physiological and biochemical mechanism underlining NH_4^+ preference of tea plants remains largely unknown.

Young shoots of tea plants, which are harvested for producing tea products, contain approximately 45 mg g^{-1} (dry base) total N, making tea plants have a large demand for N supply. N fertilizers are now widely applied in tea plantations all over the world. Yield responses to the amounts of N fertilizers are often in quadric pattern, with the highest yield at $200 - 400 \text{ kg N ha}^{-1}$ (Owuor, 2001). The effect of N

fertilization on tea quality has attracted much attention. Due to important contribution of free amino acids to the taste of green tea, it is generally considered that application of N fertilizers can improve green tea quality. However, there is no clear indication of how synthesis of individual amino acid, in particular Thea, is affected by external N supply. In a previous experiment high concentration of Arg in young shoots of tea plants was detected under saturated N supply (Okano et al., 1997). This is an important question since individual amino acid has its own specific taste (Table 2). The taste of green tea infusion is possibly affected not only by the concentration of total free acid but, more importantly, by the relative proportion of individual free amino acids.

It is generally believed that increasing N application impairs black tea quality (Owuor, 2001). The plain quality of black tea is largely determined by the concentration of theaflavins and thearubigins that are oxidation products of catechins (Biswas et al., 1973; Hazarika et al., 1984; McDowell et al., 1995; Roberts, 1958ab, 1962). However, previous results regarding the effect of N fertilization on the concentrations of catechins and their oxidation derivatives (TRs and TRs) are elusive. Hilton et al (1973) showed that N fertilization diminished (-)-EGC, (-)-EC in young shoots, whereas it either increased or decreased (-)-EGCG concentration. In contrast, a recent finding showed that total polyphenol concentration is increased by N fertilization (Venkatesan and Ganapathy, 2004). In a number of other experiments, it decreased (Cloughley, 1983; Hilton et al., 1973; Owuor and Odhiambo, 1994), did not change (Owuor et al., 1991; 2000) or even increased (Owuor et al., 1987) theaflavins and thearubigins concentrations. Contents of chlorophyll are usually increased by N fertilization (Krishnapillai and Ediriweera, 1986), which has been considered as a factor causing grassy note deteriorating black tea quality (van Lelyveld et al., 1990; van Lelyveld and Smith, 1989).

The information concerning the effect of N fertilization on aroma property of tea is limited. Early field experiment showed concentrations of aldehyde, geraniol, cis-furanoid oxide of linalool, methyl ester of salicylic acid and dihydroactinidiolide were increased, while those of linalool, pentanol, α -terpineol, beta-nerolidol and some unidentified compounds were decreased by N fertilization (Tsanava et al., 1991). Application of N fertilizers was found to increase fatty acid contents, leading to high level of undesirable aroma in black tea (Owuor et al., 1987; Owuor and Odhiambo, 1994).

4.2 Potassium, sulphur and chloride

Young tea shoots contain abundant potassium at the concentrations (15 – 25 mg g⁻¹, dry base) only next to nitrogen. Tea yield responses to potassium application were reported from various countries (Godziashvili and Peterburgsky 1985; Krishnapillai and Ediriweera, 1986; Malenga and Grice, 1991; Rahman and Jain, 1985; Sharma and Sharma, 1995). Responses of yield to K fertilization however relate to availability of K in soils, which explains examples where no yield response was obtained (Kamau et al. 1999), possibly being caused by a high K supplying capacity in soil. Analysis of soil samples taken from typical tea fields of China revealed that

more than 50% were classified as K deficient (Ruan and Hårdter, 2001; Wu and Ruan, 1994). Yield increases by application of K-fertilizers were reported in various areas of China producing different types of teas (Ruan et al., 2003; Wu and Ruan, 1994). In addition to yield response, the quality of tea is improved due to increase of total free amino acid in green tea and theaflavins and thearubigins in black tea (Ruan et al., 1998; 1999; Venkatesan and Ganapathy, 2004).

The two main sources of K-fertilizers are potassium sulphate and potassium chloride. Compare to KCl, potassium sulphate has an advantage of concomitant provision of S nutrient. The importance of S nutrition in formation of quality determining constituents (free amino acids, caffeine, and catechins) was demonstrated (Ali et al., 1997; Barbora, 1995; Ruan et al., 1998). Improved S nutrition promotes concentrations of total free amino acid (Ruan et al., 1998) and methionine (Nagendra Rao and Sharma, 2001). Concentrations of TFs, TRs and the color of black tea are positively influenced by S application (Ali et al., 1997; Barbora, 1995). Furthermore, there is a report that aroma property of tea is improved by S application (Wu and Ruan, 1994).

Very limited information on Cl nutrition, i.e. its absorption and distribution in tea plants, effect on plant growth, yield and quality is available. For most soils, inputs from various sources, such as deposition from rainwater, fertilizer application, irrigation water, dust and air pollution, supply sufficient Cl⁻ for plant growth and Cl deficiency rarely occurs (Xu et al., 2000). Similarly, either deficiency or toxicity of Cl in tea plants has rarely been reported. It is generally accepted that Cl from potassium chloride has no disadvantage in comparison with K₂SO₄ if applied to soil (Bonheure and Willson, 1992). However, there are a few examples where Cl toxicity or negative effect has been observed. Application of NH₄Cl at high amounts induces leaf injury in fields, which has been attributed to Cl toxicity (Wu and Ru, 1975). These authors showed that leaf damage occurred when Cl beyond 200 kg ha⁻¹ was applied and became more severe with increasing application amounts. Concentration of total free amino acid was significantly reduced by application of KCl compared to K₂SO₄ in a pot experiment (Ruan et al., 1998). The mechanism of the negative effect of Cl is poorly understood and has been explained as antagonistic effect on NO₃⁻ uptake and its subsequent assimilation (Ruan et al., 1998).

5 Fertilization practice in China

In China, tea plantations are established in a wide range of soil types in temperate, subtropical and tropical areas. Plant growth and product quality vary with soils of different properties (Wang et al., 1997; Yuan et al., 2000). A majority of tea is grown on soils, classified as red soils (Oxisols and Ultisols), which are highly leached and strongly acidic with low inherent fertility. Limited supply of nutrients is the major factor restricting productivity and quality improvement of tea from these soils (Han et al, 2002). A number of experiments demonstrated positive effect of fertilization of nutrients (N, P, K, Mg, S, and micronutrients Zn, Cu and Mo) on yield and quality (Tea Research Institute, 1985; Han et al., 1993; 1994; 2002; Lin et al., 1991; Ruan et al., 2002; 2003; Wu and Fang, 1992; 1994; Wu et al., 1994; Wu and Ruan, 1994).

These results highlighted the importance of a balanced application of nutrients, which however has not been currently employed widely in China. The imbalanced fertilization is characterized by increasing nitrogen application, resulting in a great surplus of N inputs over plant demands (Ruan and Wu, 2004). For example, N applied in tea fields in Zhejiang province, the largest green tea producer in China, ranged from 279 to 2104 kg ha⁻¹ year⁻¹. In Japan, up to 1470 kg N ha⁻¹ year⁻¹ on average was applied in order to produce quality green tea with elevated concentrations of free amino acids (Tachibana et al., 1995). Such huge N inputs increase production costs and impose the danger of environmental hazards (Kihou and Yuita, 1991; Kosuge et al., 1987; Toda et al., 1997).

6 Objectives of the study

Free amino acids, polyphenols and caffeine are particularly important groups of compounds contributing to taste and quality of green tea infusions. Production and accumulation of these metabolites in young shoots of tea plants are thus of uppermost importance. In this study, the influence of different nutrients (NH₄⁺, NO₃⁻, K⁺, SO₄²⁻, Cl⁻) on quality-related constituents was investigated to establish functional relationship between nutrient supply, physiological processes and product quality of tea plants.

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Chapter 1

Effect of nitrogen form and root-zone pH on growth and nutrient uptake of tea plants (*Camellia sinensis* (L.) O. Kuntze)

Abstract

Tea plants were hydroponically cultured with NH_4^+ , NO_3^- and $\text{NH}_4^++\text{NO}_3^-$ at pH 4.0, 5.0 and 6.0. Plants supplied with NO_3^- showed symptoms resembling nitrogen deficiency and grew much slower than those receiving NH_4^+ or $\text{NH}_4^++\text{NO}_3^-$ independent of root-zone pH. These plants had significantly reduced concentrations of total N and free amino acids in mature leaves as well as reduced concentration of glucose in roots compared to those with NH_4^+ or $\text{NH}_4^++\text{NO}_3^-$. Absorption of NO_3^- was considerably less than NH_4^+ and was strongly depressed when NH_4^+ and NO_3^- were simultaneously supplied. Results indicated that tea plants were adapted to NH_4^+ as the sole N source due to large capacity for NH_4^+ assimilation characterized by sharply increasing root glutamine synthetase activity and improved carbohydrate supply to roots. The poor plant growth with NO_3^- was largely associated with inefficient absorption of this N source. Biomass production was the least at pH 6.0 and largest at pH 5.0 regardless of N form. Plant growth was reduced by low root-zone pH when supplied with NO_3^- but not with NH_4^+ or $\text{NH}_4^++\text{NO}_3^-$. Decreased growth caused by inappropriate external pH was due partly to the declining absorption of nitrogen.

Introduction

Ammonium (NH_4^+) and nitrate (NO_3^-) are the most important inorganic N sources readily available to plants in soils. For many plants, NH_4^+ , when supplied solely at high concentrations is toxic and impairs plant growth (Gerendás et al., 1997; Britto and Kronzucker, 2002). However there are some plant species well adapted to this N source (Britto and Kronzucker, 2002; Brix et al., 2002; Lavoie et al., 1992; Poonnachit and Darnell, 2004; Warren and Adams, 2002). Tea is an important beverage crop widely cultivated in subtropical and tropical areas. There are some evidences that tea plants grow better with NH_4^+ than with NO_3^- nutrition (Ishigaki, 1974). Data of a ^{15}N experiment demonstrated larger absorption of NH_4^+ over NO_3^- in a short time (24 h) when both sources were supplied in nutrient solution at similar concentrations (Morita et al., 1998). Total N content in leaves is also increased by application of NH_4^+ together with a nitrification inhibitor as compared to application of NO_3^- in a soil experiment (Ruan et al., 2000). However, the mechanism behind the preference of NH_4^+ nutrition has not been clearly elucidated (Gerendás et al., 1997; Britto and Kronzucker, 2002).

Response of plant growth and nutrient absorption to N form may further vary with change of external pH (Chaillou et al., 1991; Troelstra and Blacquiere, 1986). Acidic ambient pH may diminish NH_4^+ absorption by reducing the driving force for NH_4^+ diffusion, the electrochemical gradient across the plasma membrane (Glass et al., 1997). In contrast, NO_3^- is taken up by the H^+ -cotransport system and is then likely stimulated by a low external solution pH (Miller and Smith, 1996). Tea plants

prefer acid soils and are able to grow on soils of pH below 5.0. A high proportion (44%) of China's tea fields consists of soils with pH below 4.0 (Ma et al., 2002). In some Japanese green tea fields, soil pH even declined to 2.9 (Tachibana et al. 1995). In tea plantations especially those destined for green tea production, large amounts of N fertilizers have been applied in the form of NH_4^+ or urea (Tachibana et al., 1995; Ruan and Wu, 2004), since it is recognized that green tea quality is closely correlated with the total N and free amino acid concentrations. Whilst nitrification in strongly acidic soils is expectedly low (Wickramasinghe et al., 1985), experiments however, indicated intensive nitrification and a large pool of NO_3^- in tea soils with very low pH (Hayatsu and Kosuge, 1993; Kihou and Yuita, 1991). It has not been clearly understood how the relative uptake of NH_4^+ and NO_3^- by tea plants and the consequent plant growth is affected when external pH changes. It was hypothesized that uptake of NH_4^+ by tea plants may decline whereas that of NO_3^- is favored when root-zone pH decreases, as observed in other plant species (Vessey et al., 1990; Henry and Raper; 1989; Marcus-Wyner, 1983). The present experiment was therefore conducted to investigate the effect of N form and its interaction with external pH on growth and nutrient uptake by tea plants.

Materials and Methods

Plant growth condition

Tea seedlings with 3-4 leaves germinated from seeds (cv. Longjing 43) were transplanted to $0.2 \text{ mmol L}^{-1} \text{ CaSO}_4$ solution for five days and then transferred to 1/3 strength nutrient solution containing three different sources of nitrogen (NH_4^+ , NO_3^- and 50% NH_4^+ + 50% NO_3^-) for one week. Strength of the nutrient solution was increased stepwise to 2/5 (week 2 and 3), 1/2 (week 4), 3/4 (week 5 to 12) and full (week 13 to 20). The composition of full strength nutrient solution contained macronutrients (mmol L^{-1}) N 1.7, P 0.07, K 0.67, Ca 0.53, Mg 0.67 and micronutrients ($\mu\text{mol L}^{-1}$) Zn 0.67, Cu 0.13, Mn 1.0, B 7.0, Mo 0.33, and Fe 4.2 as EDTA salt. Aluminium was added to the nutrient solution at 0.07 mM for its beneficial effect (Konishi et al., 1985). To inhibit any potential nitrification in the nutrient solution (Padgett and Leonard, 1993), nitrification inhibitor 3,4-dimethyl pyrazole phosphate (DMPP) at 1% of the N amount was added. The pH of nutrient solutions were continuously titrated to 4.0, 5.0 and 6.0 for each of N sources with H_2SO_4 and NaOH by means of pH stat systems. Each pot contained 4 L nutrient solution with three seedlings and was thinned to two at week 15. The nutrient solution was replaced every one week. The experiment was conducted for 20 weeks from May to September. Plants were placed in a naturally lit glasshouse provided with additional light (SON-T AGRO 400 W, Philips) to ensure minimum intensity of $220 \mu\text{mol m}^{-2} \text{ s}^{-1}$ at canopy level until week 4, thereafter lit solely by natural light. Maximum air temperature in the glasshouse was approximately 34 °C during daytime and minimum 20 °C at night. Relative humidity was maintained around 70% by a humidifier. To facilitate branching, apex buds were removed after the first-round growth (week 8). Young shoots of one bud and two leaves (as for tea product) were plucked thereafter. The plants were destructively sampled at week 20, frozen quickly in liquid N_2 and freeze dried. The relative growth rate (RGR) over the whole

experimental duration (20 week) is calculated according to the equation ($RGR = [\ln(W_2) - \ln(W_1)] / (t_2 - t_1)$), where W_1 and W_2 represent whole plant dry weight (g) at time t_1 and t_2).

Chlorophyll content

Chlorophyll contents of mature leaves were measured by a portable chlorophyll meter (Minolta SPAD-502, Osaka) at week 4 and 9. For each plant 4-12 leaves from the upper canopy were randomly selected and average readings were recorded as one replicate. The chlorophyll meter readings were calibrated ($r^2 = 0.912$, $p < 0.0001$) with chlorophyll contents measured by the method of Arnon (1949) from a parallel experiment supplied with NH_4^+ or NO_3^- under similar growth conditions.

Specific absorption rates of NH_4^+ and NO_3^-

Specific absorption rates of NH_4^+ and NO_3^- were determined by measuring their depletion in the nutrient solutions for four times, two over one-week intervals at week 10 and 11 and another two over 5-day intervals at week 18 and 19, respectively. Concentrations of NH_4^+ and NO_3^- in the nutrient solutions were determined by the indophenol blue method and the nitrate ion-selective electrode (Ionplus combination, Orion Research Inc., Beverly, USA), respectively (Mulvaney, 1996). Absorption rates were expressed on a per root dry weight basis calculated from data at the final sampling (week 20) and at the beginning (week 1) assuming a constant growth rate per week. Average rates from the four measurements are reported here.

Enzyme assay

Glutamine synthetase (GS, EC 6.3.1.2) in fibrous roots and young expanding leaves (the third leaf from the bud) was extracted with a buffer solution (pH 7.5) containing 50 mmol L⁻¹ Tris, 5 mmol L⁻¹ EDTA, 5 mmol L⁻¹ dithioerythritol at a ratio of 10 ml g⁻¹ FW and 5% (w/v) PVPP using a Potter S homogenizer cooled with ice (Gerendás et al., 1998). Extracts were centrifuged at 12 000 g for 10 min and the crude enzyme was used for activity assay. GS activity was determined by the synthetase assay (Magalhaes and Huber, 1989) and a 30-min incubation time was adopted. One unit enzyme activity corresponded to the formation of 1 μ mol γ -glutamyl hydroxamate per gram fresh material per min.

Nutrient concentrations

Plant samples were ashed at 500°C and measured for P, K, Mg, Ca, Al by Induced Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Model IRIS-AP, Thermo Jarrel Ash Corp., USA). Total N was determined in an elemental analyzer (Carlo Erba, Milano, Italy).

Determination of free amino acids, soluble reduced sugars and anions

Free amino acids in plant samples of finely ground powder were extracted with H₂O (1/50, w/v) in 100°C water bath for 5 min and analyzed as o-phthalaldehyde derivatives on a reversed phase C₁₈ column (Hypersil ODS, 3 µm, 250 mm x 4.6 mm) using an automated HPLC system (Gerendás et al., 1998). Standards were prepared from authentic compounds and norvaline was used as an internal standard. Soluble reduced sugars, NO₃⁻ and organic anions in plant samples were extracted with chloroform: methanol (3:7, v/v) as previously described (Lohaus et al., 2000) and measured by ion chromatography (DX 300, Dionex, Idstein, Germany).

Statistics

Data were subjected to two-way analysis of variance to examine the effects of N form, pH and their interaction.

Results

Plant growth and biomass production

Plants supplied with NO₃⁻ displayed yellowish leaves resembling symptoms of nitrogen deficiency from the very beginning of the experiment. Their leaves had lower contents of chlorophyll than plants receiving other two N sources throughout the experimental period (Figure 1, A and B). Root morphology of plants was also quite different among N sources at pH 4.0 and pH 5.0 at the early stage. The plants with NH₄⁺ and NH₄⁺+NO₃⁻ grew well with long and white seminal roots while new root development in NO₃⁻ supplied plants was much slower with shorter seminal roots. The biomass production was significantly less in NO₃⁻ than in NH₄⁺- or NH₄⁺+NO₃⁻-fed plants and was not statistically different between the latter two N sources (Table 1).

Table 1. Biomass production (g pot⁻¹) of tea plants grown with different N forms and root-zone pH. Data are means ± standard deviations (n=4).

Treatment		Whole plant	Shoot	Root
N form	pH			
NH ₄ ⁺	4.0	15.58±2.95	11.79±2.36	3.79±0.82
	5.0	19.51±1.85	15.80±1.60	3.71±0.42
	6.0	11.24±1.94	8.76±1.88	2.49±0.12
NO ₃ ⁻	4.0	6.47±1.80	4.64±1.60	1.83±0.22
	5.0	11.42±1.14	8.77±1.08	2.65±0.22
	6.0	4.10±1.76	2.91±1.49	1.18±0.33
NH ₄ ⁺ +NO ₃ ⁻	4.0	14.58±3.32	11.70±2.85	2.88±0.58
	5.0	16.78±1.45	13.85±1.60	2.93±0.18
	6.0	12.77±1.58	9.82±1.47	2.95±0.20
ANOVA (F value)				
Factor	N form	55.63**	50.19**	49.52**
	pH	29.62**	28.64**	20.52**
	N form × pH	1.33	0.80	7.72**

*: p<0.05; **: p<0.01

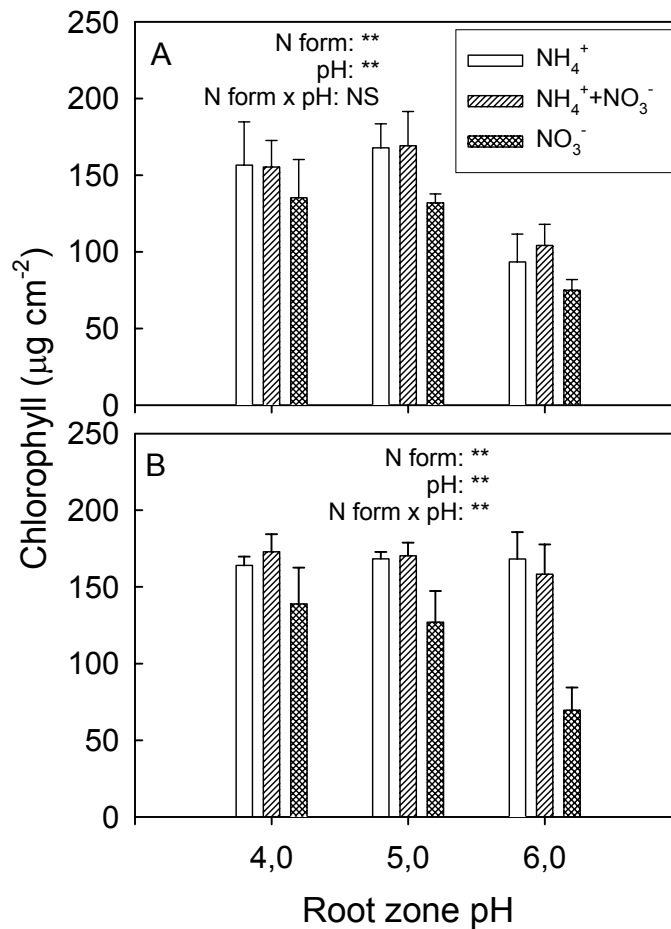


Figure 1. Chlorophyll contents in mature leaves of tea plants grown with different N forms and root-zone pH (A) 4 and (B) 9 weeks after onset of the treatments. Bars are standard deviations (n=4). Results of two-way ANOVA are indicated.

** : $p < 0.01$, * : $p < 0.05$, NS: not significant.

Response of plant growth to root-zone pH varied with the N form supplied. Shortly after treatment onset, plants exposed to pH 6.0 had stunted and brown seminal roots with all N sources and showed chlorotic leaves containing considerably less chlorophyll than those of the other two pH treatments at the early growth stage (week 4) (Figure 1, A). After a prolonged time, those plants supplied with NH₄⁺ or NH₄⁺+NO₃⁻ slowly recovered and attained comparable chlorophyll levels in the leaves (Figure 1, B), whereas those supplied with NO₃⁻ remained chlorotic until the final sampling (week 20) (data not shown). Shoot and whole plant biomass production was the largest at pH 5.0 and the least at pH 6.0 for all N sources (Table 1). Shoot growth of NO₃⁻- and NH₄⁺-supplied plants, but not those of NH₄⁺+NO₃⁻ supplied plants, were reduced at pH 4.0 compared to pH 5.0. Root growth was also strongly reduced at pH 4.0 in plants supplied with NO₃⁻ but was unaffected in plants receiving NH₄⁺ or NH₄⁺+NO₃⁻. Whole plant biomass production decreased significantly at pH 4.0 for NO₃⁻-fed plants but only slightly ($p > 0.05$) for NH₄⁺- or NH₄⁺+NO₃⁻-supplied plants.

GS enzyme activity

The activity of GS in fibrous roots and young leaves was increased by the provision of NH_4^+ compared to NO_3^- (Figure 2). Plant roots receiving NH_4^+ + NO_3^- had an intermediate level of GS activity, being substantially greater than those provided with NO_3^- but slightly lower than in plants receiving NH_4^+ (data not shown). The effect of root-zone pH on GS activity was insignificant. However there was a significant interaction between N form and root-zone pH showing a tendency of decreasing leaf enzyme activity at pH 4.0.

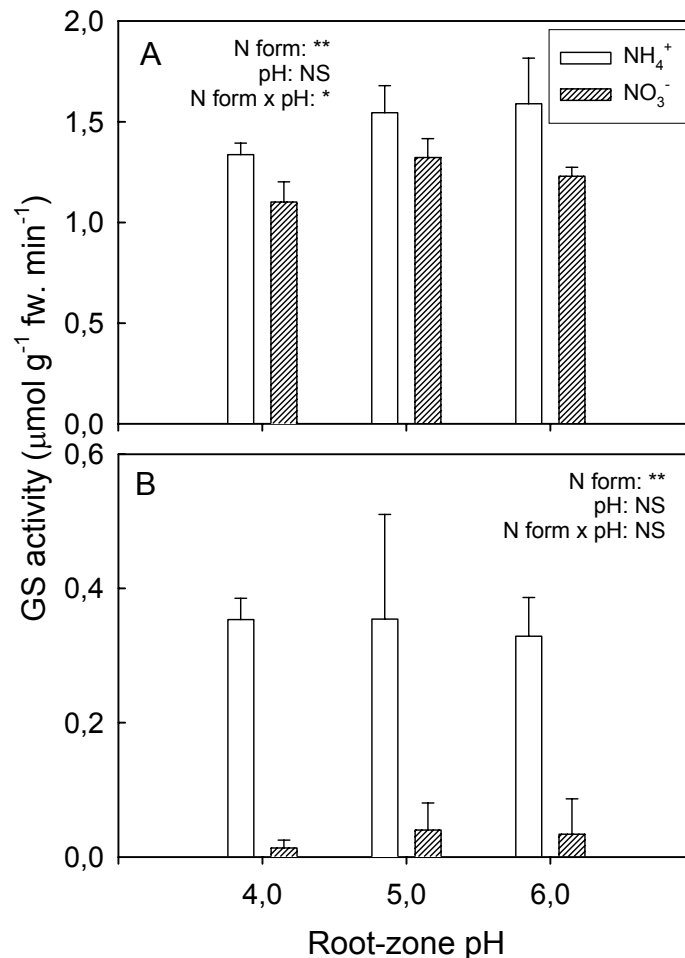


Figure 2. Glutamine synthetase activity in young expanding leaves (A) and fibrous roots (B) of tea plants grown with different N forms and root-zone pH. Bars are standard deviations (n=3-4). Results of two-way ANOVA are indicated. **: $p < 0.01$, *: $p < 0.05$, NS: not significant.

Nitrogen absorption, concentrations of cations and anions

Absorption of NH_4^+ was much larger than NO_3^- independent of root-zone pH (Table 2). When NH_4^+ and NO_3^- were individually supplied, NH_4^+ absorption rates were 2.7, 2.0, and 3.5 fold greater than NO_3^- at pH 4.0, 5.0 and 6.0, respectively. The differences became larger when NH_4^+ and NO_3^- were co-provided, being 6.2, 6.5 and 16.2 fold greater for NH_4^+ than for NO_3^- . This is because nitrate uptake was substantially depressed by co-provision of NH_4^+ , being 3.0, 5.1 and 6.8 fold smaller in plants co-provided than in plants solely supplied with NO_3^- at pH 4.0, 5.0, and 6.0,

respectively. Absorption of NH_4^+ and NO_3^- when individually supplied was the largest at pH 5.0 and least at pH 6.0 (Table 2). NH_4^+ uptake differed insignificantly between pH 5.0 and pH 4.0 whereas NO_3^- absorption was reduced by 37% ($p < 0.05$) at pH 4.0 compared to at pH 5.0. The absorption of NH_4^+ or NO_3^- was only marginally affected by root-zone pH when they were simultaneously supplied. With regard to N forms, the rate for total N ($\text{NH}_4^+ + \text{NO}_3^-$) uptake was in the order $\text{NH}_4^+ > \text{NH}_4^+ + \text{NO}_3^- > \text{NO}_3^-$ and $\text{pH } 5.0 > \text{pH } 4.0 > \text{pH } 6.0$ with respect to root-zone pH. The relative growth rate (RGR) closely correlated with the total N absorption rate, which may be described by an exponential equation (Figure 3).

Table 2. NH_4^+ and NO_3^- specific absorption rates of tea plants grown with different N forms and root-zone pH. Data are means \pm standard deviations ($n=4$).

Treatment		Specific absorption rate ($\mu\text{mol g}^{-1}$ root dw day $^{-1}$)		
N form	pH	NH_4^+	NO_3^-	$\text{NH}_4^+ + \text{NO}_3^-$
NH_4^+	4.0	165.9 \pm 21.8		165.9 \pm 21.8
	5.0	194.2 \pm 23.8		194.2 \pm 23.8
	6.0	151.2 \pm 27.0		151.2 \pm 27.0
NO_3^-	4.0		60.9 \pm 9.4	60.9 \pm 9.4
	5.0		96.3 \pm 13.5	96.3 \pm 13.5
	6.0		43.6 \pm 2.3	43.6 \pm 2.3
$\text{NH}_4^+ + \text{NO}_3^-$	4.0	124.8 \pm 39.2	20.1 \pm 7.6	144.9 \pm 46.0
	5.0	121.2 \pm 40.7	18.7 \pm 11.8	139.9 \pm 51.1
	6.0	103.6 \pm 13.7	6.4 \pm 3.2	110.0 \pm 16.6
ANOVA (F-value)				
Factor	N form	20.31**	200.71**	41.90**
	pH	2.16	26.21**	6.72**
	N form x pH	0.66	12.38**	0.61

*: $p < 0.05$; **: $p < 0.01$

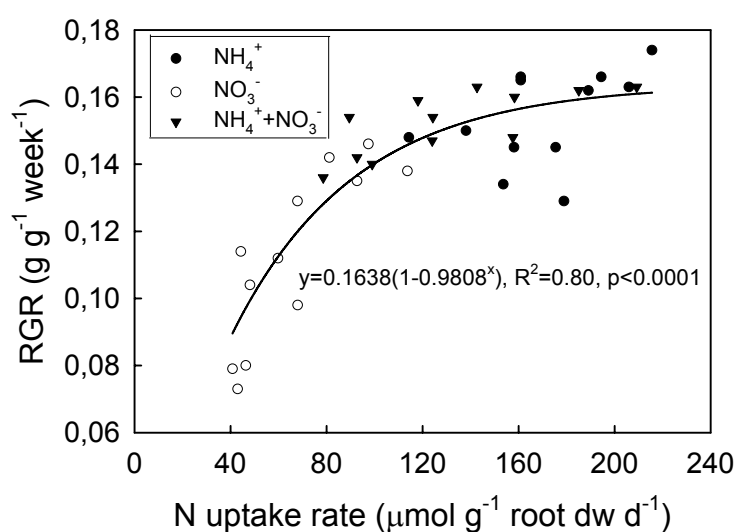


Figure 3. Relationship of relative growth rate (RGR) and specific N absorption rate of tea plants grown with different N forms and root-zone pH.

Table 3. Concentrations of elements (mg g⁻¹) in mature leaves and fibrous roots of tea plants grown with different N forms and root-zone pH.
Data are means ± standard deviations (n=4).

Treatment		N	P	K	Ca	Mg	Al
N form	pH						
<i>Mature leaves</i>							
NH ₄ ⁺	4.0	46.6±5.7	5.3±1.1	7.5±1.2	4.1±0.3	2.5±0.3	1.3±0.1
	5.0	46.1±2.9	3.9±0.7	6.9±0.4	4.0±0.5	3.0±0.4	1.6±0.2
	6.0	44.6±4.2	3.9±0.6	6.9±1.0	3.9±0.2	3.3±0.3	0.5±0.2
NO ₃ ⁻	4.0	30.8±3.1	3.0±1.1	9.3±0.5	4.8±0.4	3.0±0.3	1.3±0.6
	5.0	30.6±1.5	5.9±0.9	9.8±0.9	5.0±0.5	3.7±0.3	1.3±0.3
	6.0	26.6±1.3	2.7±0.6	13.7±3.5	8.4±2.9	6.0±1.8	0.1±0.0
NH ₄ ⁺ +NO ₃ ⁻	4.0	44.4±4.9	5.3±1.1	8.4±1.2	4.1±0.2	2.8±0.1	1.6±0.3
	5.0	41.0±2.4	5.0±0.5	8.9±1.1	4.1±0.3	3.2±0.3	1.8±0.5
	6.0	41.4±3.1	3.8±0.7	8.6±0.7	3.9±0.5	3.1±0.3	0.5±0.2
ANOVA (F-value)							
<i>Factor</i>	N form	72.81**	2.97	21.56**	15.68**	14.18**	4.21*
	pH	2.26	9.40**	3.19*	3.98*	13.87**	42.38**
	N form x pH	0.47	7.72**	4.26*	5.51**	5.77**	0.52
<i>Fibrous root</i>							
NH ₄ ⁺	4.0	39.7±7.3	11.3±2.1	12.9±1.3	2.2±0.4	2.1±0.4	0.29±0.01
	5.0	35.3±2.4	9.0±1.4	14.0±2.6	2.8±0.2	3.1±0.4	0.26±0.11
	6.0	42.5±2.3	12.5±3.1	13.6±1.3	2.7±0.3	3.3±0.5	0.32±0.07
NO ₃ ⁻	4.0	23.2±1.5	12.4±1.8	13.4±2.4	2.4±0.2	3.1±0.3	0.88±0.19
	5.0	22.0±1.5	19.0±1.0	13.5±2.2	3.2±0.1	5.3±0.3	0.85±0.15
	6.0	19.1±3.1	17.4±1.9	15.3±3.3	3.5±0.9	7.4±0.9	0.59±0.13
NH ₄ ⁺ +NO ₃ ⁻	4.0	30.1±1.4	13.6±1.9	15.9±2.7	2.2±0.2	2.7±0.4	0.42±0.08
	5.0	30.2±1.5	14.5±1.7	16.3±2.7	2.7±0.2	3.5±0.5	0.49±0.14
	6.0	29.5±1.7	14.6±1.5	17.9±2.4	2.8±0.2	4.4±0.5	0.25±0.06
ANOVA (F-value)							
<i>Factor</i>	N form	98.64**	23.38**	5.94**	6.33**	71.08**	55.82**
	pH	1.13	5.18*	1.23	12.20**	68.74**	5.99**
	N form x pH	3.19*	5.83**	0.26	0.60	11.28**	2.79*

*: p<0.05; **: p<0.01

Mature leaves and roots of plants supplied with NH₄⁺ contained significantly larger total N concentrations than those supplied with NO₃⁻ whereas NH₄⁺+NO₃⁻ supplied plants had intermediate levels (Table 3). Specific nitrogen absorption rate closely correlated with total N concentration in mature leaves (r=0.82, P<0.001) and roots (r=0.72, P<0.001). Provision of NO₃⁻ (NO₃⁻ or NH₄⁺+NO₃⁻) significantly increased NO₃⁻ concentrations in roots and slightly in mature leaves (Table 4). Both total N and NO₃⁻ concentrations were unaffected by root-zone pH. Concentrations of cations K, Ca and Mg in mature leaves were considerably higher in plants supplied with NO₃⁻ than those with NH₄⁺. A similar effect of NO₃⁻ nutrition was observed for concentrations of Ca and Mg in roots, but not K which was higher in NH₄⁺+NO₃⁻ supplied plants. Low root-zone pH reduced leaf Mg, root Ca and Mg concentrations independent of N forms supplied, and leaf K and Ca concentrations in plants receiving NO₃⁻. Root Al concentration was higher in NO₃⁻ supplied plants than in

NH₄⁺ or NH₄⁺+NO₃⁻ supplied plants whereas leaf Al concentration was not influenced by N form, except at pH 6.0 where it was lower in plants receiving NO₃⁻. Increasing the root-zone pH diminished the concentrations of Al in leaves and roots except in plants supplied with NH₄⁺. Concentration of P in roots increased when plants were supplied with NO₃⁻ at pH 5.0 and pH 6.0 whereas in mature leaves no consistent treatment effect was observed.

Oxalate was the predominant organic anion in roots and mature leaves, followed by citrate and malate (Table 4). Their concentrations were larger in roots than in mature leaves and were generally larger at pH 6.0 than at other pH treatments and in plants receiving NO₃⁻ than in plants supplied with NH₄⁺ or NH₄⁺+NO₃⁻. This observation became more evident in roots than in mature leaves.

Table 4. Concentrations of nitrate ($\mu\text{g g}^{-1}$) and organic anions (mg g^{-1}) in mature leaves and fibrous roots of tea plants grown with different N forms and root-zone pH. Data are means \pm standard deviations of (n=4)

Treatment		Mature leaves				Fibrous roots			
N form	pH	NO ₃ ⁻	Citrate	Malate	Oxalate	NO ₃ ⁻	Citrate	Malate	Oxalate
NH ₄ ⁺	4.0	7 \pm 1	0.01 \pm 0.01	0.08 \pm 0.03	0.8 \pm 0.2	9 \pm 3	0.27 \pm 0.08	0.22 \pm 0.17	1.6 \pm 0.5
	5.0	8 \pm 1	0.05 \pm 0.03	0.18 \pm 0.03	1.2 \pm 0.3	11 \pm 7	0.50 \pm 0.23	0.32 \pm 0.15	2.3 \pm 0.5
	6.0	9 \pm 1	0.20 \pm 0.09	0.22 \pm 0.05	1.3 \pm 0.3	10 \pm 2	0.44 \pm 0.10	0.26 \pm 0.08	3.5 \pm 0.5
NO ₃ ⁻	4.0	18 \pm 14	0.22 \pm 0.19	0.26 \pm 0.02	1.3 \pm 0.3	322 \pm 135	0.36 \pm 0.09	0.14 \pm 0.06	3.5 \pm 1.2
	5.0	13 \pm 3	0.70 \pm 0.61	0.26 \pm 0.04	1.6 \pm 0.3	299 \pm 85	0.71 \pm 0.18	0.14 \pm 0.05	5.6 \pm 0.9
	6.0	10 \pm 5	1.47 \pm 0.84	0.60 \pm 0.23	4.7 \pm 0.9	310 \pm 145	1.21 \pm 0.21	0.25 \pm 0.08	12.2 \pm 3.0
NH ₄ ⁺ +NO ₃ ⁻	4.0	10 \pm 3	0.05 \pm 0.04	0.18 \pm 0.05	1.1 \pm 0.1	293 \pm 77	0.50 \pm 0.16	0.33 \pm 0.10	3.3 \pm 0.9
	5.0	28 \pm 15	0.08 \pm 0.03	0.24 \pm 0.07	1.3 \pm 0.3	401 \pm 70	0.75 \pm 0.23	0.37 \pm 0.17	3.7 \pm 0.8
	6.0	18 \pm 16	0.19 \pm 0.02	0.25 \pm 0.04	1.0 \pm 0.2	303 \pm 153	0.68 \pm 0.29	0.33 \pm 0.10	6.9 \pm 1.1
ANOVA (F-value)									
Factor	N form	4.02*	15.65**	19.74**	58.26**	43.49**	11.20**	5.99**	41.41**
	pH	0.95	7.14**	14.35**	39.79**	0.38	14.17**	0.63	43.96**
	N form x pH	1.80	3.34*	4.75**	32.38	0.64	4.88**	0.70	8.12**

*: p<0.05; **: p<0.01

Free amino acids and soluble reduced sugars

Mature leaves of NH₄⁺ supplied plants contained substantially increased concentrations of free amino acids compared to those given NO₃⁻ and those with the mixed N forms contained intermediate levels (Table 5). The profile of free amino acids was also largely changed by the application of different N forms. The predominant amino acid in the plants supplied with NO₃⁻ was glutamic acid (Glu), followed by theanine (Thea, N⁵-ethyl-glutamine), aspartic acid (Asp), and glutamine (Gln) whereas in NH₄⁺ supplied plants, Thea and arginine (Arg) predominated, followed by Glu, Gln and Asp. Root-zone pH and its interaction with N-form affected none of the major amino acids mentioned above.

Fibrous roots of the plants supplied with NH₄⁺+NO₃⁻ or NH₄⁺ contained similar concentrations of glucose, which were significantly larger than in NO₃⁻-fed plants (Table 6). Root fructose concentration was greater in plants receiving NH₄⁺+NO₃⁻ than in plants receiving NH₄⁺ or NO₃⁻. In contrast, mature leaves of plants receiving NH₄⁺ had lower glucose concentration than NO₃⁻- or NH₄⁺+NO₃⁻ supplied plants. A

similar observation was made for the concentration of fructose in leaves but the statistical significance was dependent on pH owing to interaction between N form and pH. Sucrose concentrations in roots and mature leaves were unchanged by N form or root-zone pH.

Table 5. Concentrations of free amino acids ($\mu\text{mol g}^{-1}$) in mature leaves of tea plants grown with different N forms and root-zone pH. Data are means (n=4).

Treatment		Amino acid						
N form	pH	Thea	Gln	Arg	Glu	Asp	Others	Sum
NH ₄ ⁺	4.0	35.51	19.46	48.89	25.09	16.91	16.74	162.60
	5.0	24.43	15.80	42.52	28.48	16.68	17.51	145.43
	6.0	49.08	21.49	34.60	30.64	17.63	16.33	169.78
NO ₃ ⁻	4.0	10.43	3.84	0.88	21.30	9.50	8.13	54.07
	5.0	5.75	4.42	1.72	20.12	9.38	5.16	46.54
	6.0	6.61	4.20	2.50	22.98	6.59	6.26	49.13
NH ₄ ⁺ +NO ₃ ⁻	4.0	25.36	6.05	16.63	22.36	12.42	18.61	101.43
	5.0	7.64	4.11	8.41	20.76	13.69	11.36	65.98
	6.0	19.22	6.83	11.11	25.91	14.51	11.63	89.20
ANOVA (F-value)								
Factor	N form	35.47**	19.32**	18.06**	7.80**	29.13**	17.84**	31.44**
	pH	1.83	0.56	0.41	2.66	0.06	2.04	1.17
	N form x pH	1.70	0.23	0.27	0.51	1.03	1.03	0.25

*: p<0.05; **: p<0.01

Table 6. Carbohydrates (mg g⁻¹) in mature leaves and fibrous roots of tea plants grown with different N forms and root-zone pH. Data are means \pm standard deviations (n=4).

Treatment		Mature leaves			Fibrous roots		
N form	pH	Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
NH ₄ ⁺	4.0	7.9 \pm 2.9	5.3 \pm 1.7	68.3 \pm 2.0	2.7 \pm 0.6	1.8 \pm 1.1	18.4 \pm 10.2
	5.0	7.8 \pm 3.3	5.9 \pm 2.8	68.7 \pm 3.3	2.6 \pm 0.3	1.6 \pm 0.8	24.6 \pm 11.9
	6.0	8.8 \pm 2.7	6.4 \pm 1.6	74.5 \pm 7.8	3.0 \pm 0.8	2.4 \pm 2.2	19.5 \pm 5.4
NO ₃ ⁻	4.0	15.4 \pm 3.3	11.3 \pm 3.0	68.1 \pm 2.4	1.6 \pm 0.2	1.5 \pm 0.5	21.1 \pm 9.1
	5.0	11.6 \pm 2.4	8.0 \pm 1.3	68.7 \pm 6.6	1.3 \pm 0.5	1.4 \pm 1.0	19.3 \pm 7.4
	6.0	11.0 \pm 3.9	6.7 \pm 3.1	62.0 \pm 8.6	1.4 \pm 0.4	1.3 \pm 0.7	23.8 \pm 8.1
NH ₄ ⁺ +NO ₃ ⁻	4.0	11.8 \pm 2.5	7.8 \pm 2.3	72.0 \pm 5.4	3.5 \pm 1.2	2.9 \pm 0.4	21.7 \pm 7.0
	5.0	15.2 \pm 4.0	10.6 \pm 2.4	64.8 \pm 5.2	3.1 \pm 0.2	2.7 \pm 0.7	27.0 \pm 8.8
	6.0	9.1 \pm 1.7	7.0 \pm 1.8	60.9 \pm 9.8	2.7 \pm 0.4	2.7 \pm 0.7	23.5 \pm 4.2
ANOVA (F value)							
Factor	N form	7.66**	5.78**	1.96	25.19**	5.37*	0.52
	pH	1.75	1.58	1.06	0.48	0.19	0.45
	N form x pH	0.47	2.48	2.84*	2.44	0.32	0.45

*: p<0.05; **: p<0.01

Discussion

Effect of pH on plant growth and nutrient uptake

Plant biomass production was the largest at pH 5.0 regardless of the N forms

supplied, consistent with the observation that the most suitable pH for tea ranges from 5.0 to 5.6 (Othieno, 1992). Growth was reduced more strongly at pH 6.0 than at pH 4.0, indicating that tea plants were sensitive to high external pH. Whilst pH 6.0 is considered as more or less the optimal value for many plants, detrimental effects have been reported for some plant species. For instance, root elongation of *Lupinus angustifolius* is markedly decreased by pH \geq 6.0 (Tang et al., 1996). The mechanism for the negative effect of high pH has not been clearly understood (Marschner, 1995), but for *L. angustifolius* it is neither related to proton production, cell wall acidification nor the change of membrane integrity of cortical cells (Tang et al., 1996; Yu et al., 2001). Alternatively, the effect of pH on plant growth has been frequently interpreted as a result of reduced nutrient absorption by inappropriate external pH (Brix et al., 2002; Henry and Raper, 1989; Vessey et al., 1990). In the present experiment, poor plant growth at pH 6.0 did not appear to be directly associated with absorption of macronutrients such as K, Mg, Ca or micronutrients Zn, Fe and Mn (data not shown). Their concentrations in plant tissues were either promoted or unaffected compared to pH 5.0. At pH 6.0, specific absorption rates of NH_4^+ and NO_3^- diminished by 22.1% and 54.7%, respectively, compared to pH 5.0, suggesting that growth inhibition was due partly to the declining absorption of N. Nevertheless, biomass production of plants individually supplied with NH_4^+ and NO_3^- was reduced by 42.4% and 64.1% at pH 6.0, respectively. These data showed that plant growth was reduced more than N absorption, suggesting that factors other than N absorption might have also played roles. However, compared to the drastic effect on N absorption, high root-zone pH only marginally affected total N concentrations in mature leaves and roots. This is possibly explained by the fact that plant growth had conformed to N absorption, rendering comparable total tissue N concentrations of plants grown in different root-zone pH within the same N sources.

It has been previously demonstrated that absorption of NO_3^- is favored when root-zone pH decreases (Vessey et al., 1990; Henry and Raper, 1989; Marcus-Wyner, 1983). The present data showed that plant growth and N absorption at low root-zone pH were not improved by the supply of NO_3^- . In contrary, NO_3^- supplied plants displayed higher sensitivity to low root-zone pH. The absorption rate of NO_3^- was diminished by 37% ($p < 0.05$) while only 15% ($p > 0.05$) for NH_4^+ at pH 4.0 compared to pH 5.0 when the two N forms were individually supplied. Furthermore, root biomass production of plants receiving NO_3^- at pH 4.0 was reduced by 31% whereas those of NH_4^+ or $\text{NH}_4^+ + \text{NO}_3^-$ supplied plants were unaffected and their whole plant biomass decreased only slightly ($p > 0.05$). This may be particularly important for tea plants, which prefer NH_4^+ (see below) despite strong acidification in the rhizosphere induced by this N form (Ruan et al., 2000). If unadjusted, the pH of NH_4^+ and $\text{NH}_4^+ + \text{NO}_3^-$ nutrient solutions could drop more than one pH unit within a day. This is in accordance with the observation that NH_4^+ -preferential or -tolerant plants are generally acid tolerant (Britto and Kronzucker, 2002). The growth of plants supplied with $\text{NH}_4^+ + \text{NO}_3^-$ differing to a lesser extent between pH 5.0 with other root-zone pH than those found in plants supplied with NH_4^+ was principally attributed to the smaller biomass production in the former plants. Low root-zone pH reduced concentrations of K, Ca and Mg in plant tissues. This is explained as the result of a diminished electro-chemical gradient across the plasma membrane generated by

H⁺-efflux pumps at lower external pH (Marschner, 1995).

Effects of NH₄⁺ and NO₃⁻ on tea plant growth and nutrient uptake

NH₄⁺ and NO₃⁻ are the most important inorganic N sources to plants, but show different effects. While NH₃ toxicity is considered as a universal biological phenomenon (Britto and Kronzucker, 2002), results of this study showed preferential uptake of and superior plant growth with NH₄⁺, clearly indicating that tea plant is well adapted to this N source. This was independent of root-zone pH. This conclusion is further supported by the observation of sharply increasing GS activity in plants supplied with NH₄⁺. NH₄⁺ is assimilated principally in roots via glutamine synthetase - glutamine-oxoglutarate aminotransferase (GS-GOGAT) pathway (Huppe and Turpin, 1994). Such a response of GS activity to N form provides essential capacity for tea plants to assimilate the majority of NH₄⁺ in the roots to avoid any excessive accumulation to lethal concentrations (Magalhaes and Huber, 1989; Raab and Terry, 1995). Meanwhile, the assimilation of NH₄⁺ requires substantial ketoglutarate obtained from glucose (finally sucrose transported from leaves) through the glycolytic and Krebs cycle pathways (Huppe and Turpin, 1994). It has been proposed that tolerance of plants to NH₄⁺ nutrition is associated with carbohydrate status in roots (Schortemeyer et al., 1997). The larger concentration of glucose in roots of tea plants supplied with NH₄⁺ (NH₄⁺ or NH₄⁺+NO₃⁻) than with NO₃⁻ provides additional evidence for the tea plants' adaptation to NH₄⁺ nutrition. In another experiment, higher concentrations of glucose in roots supplied with increasing N (mainly NH₄⁺) were observed (Chapter 3). No detection was made of any difference in the maximum photosynthetic rates (per leaf area) between NH₄⁺ and NO₃⁻ supplied plants at pH 5.0 from a parallel experiment (data not shown). But NH₄⁺ treated plants had greater numbers of leaflet therefore larger total leaf area than NO₃⁻ supplied plants, indicating a stronger source size in plants receiving NH₄⁺. Since sucrose concentrations were at similar levels in the roots with different N forms, higher glucose concentration there suggests possible larger transport of sucrose from leaves and faster turnover from sucrose to glucose when demand for carbon skeletons is high under NH₄⁺ nutrition. Alternatively low glucose level could be a result of greater consumption under NO₃⁻ nutrition. The metabolic cost for absorption and utilization of NO₃⁻ is believed far greater than for NH₄⁺ because NO₃⁻ must first be reduced to NO₂⁻ and then to NH₄⁺ (Bloom et al., 1992). However this seems unlikely to be of significant importance in the present study since compared to NH₄⁺, absorption and assimilation of NO₃⁻ were much smaller. Lower concentrations of glucose and fructose in the mature leaves of plants supplied with NH₄⁺ are possibly explained by a diversion of photosynthetic carbon to the synthesis of amino acids and proteins (reflected by higher free amino acids and total N contents) (Raab and Terry, 1995). The large capacity for NH₄⁺ assimilation of tea plants was further demonstrated by the abundant amides (Thea and Gln) in the roots supplied with NH₄⁺, which were 3-25 and 16-44 fold larger than in NO₃⁻ supplied plants (Chapter 2). Overall data indicated that tea plants have a high capacity to assimilate NH₄⁺ in their roots by sharply increasing key enzyme activity and improving carbohydrate supply to the roots.

Absorption of NH_4^+ was larger in plants receiving NH_4^+ than in those supplied with $\text{NH}_4^++\text{NO}_3^-$, corresponding well to the different total N concentrations in roots and mature leaves between these two N forms. Also concentrations of free amino acids, particularly Thea, Arg and Gln, in the mature leaves of $\text{NH}_4^++\text{NO}_3^-$ supplied plants were lower than those getting NH_4^+ . These data suggest that N supply in plants receiving $\text{NH}_4^++\text{NO}_3^-$ was not saturated when NO_3^- contributed only a minimal fraction to the overall N uptake. These observations were especially evident at pH 5.0 and provide an explanation for the smaller biomass production of these plants compared to those receiving only NH_4^+ at this specific root-zone pH. Compared to plants solely supplied with NO_3^- , NO_3^- absorption in $\text{NH}_4^++\text{NO}_3^-$ supplied plants was inhibited by the simultaneously supplied NH_4^+ . The inhibitory effect of NH_4^+ on NO_3^- absorption was previously reported and is attributed to the down-regulation of transporters for NO_3^- by NH_4^+ and/or their downstream metabolites at transcriptional and post-transcriptional levels (Touraine et al., 2001; Glass et al., 2002). In the present experiment the depression of NO_3^- absorption rates by NH_4^+ occurred concomitantly with a substantial increase of many free amino acids particularly Thea, Arg, and Gln in leaves and roots (Chapter 2). This finding is consistent with previous results indicating that net NO_3^- uptake is depressed by elevated intracellular concentrations of amino acids. However, it is still debatable whether NH_4^+ and/or its downstream metabolites the amino acids are the effectors and which amino acid is the most effective (Glass et al., 2002).

The present work revealed reduced plant growth with NO_3^- compared to NH_4^+ . It has been postulated that poor growth of NO_3^- - compared to NH_4^+ -fed plants is possibly due to inducing a deficiency of other nutrients (Warren and Adams, 2002). In the present experiment, the concentrations of macro- (P, K, Mg and Ca) and micro-nutrients (Fe, Zn, Cu, and Mn, data not shown) in the plants fell in normal ranges and did not appear to be directly associated with poor growth. Compared to NH_4^+ supplied plants, plants receiving NO_3^- contained even higher concentrations of K, Mg and Ca, which is consistent with earlier findings in tea (Ruan et al., 2000) and in other plants (reviewed by Britto and Kronzucker, 2002). Roots of plants supplied with NO_3^- contained significantly larger Al concentrations than those with NH_4^+ , agreeing with the finding previously reported in Al sensitive plants (Klotz and Horst, 1988). In this context, accumulation of Al in roots could not account for the poor growth with NO_3^- nutrition since Al is considered as a beneficial element stimulating the growth of tea plants (Konishi et al., 1985). A supplemental experiment also showed that omission of Al from the nutrition solution did not improve the growth of NO_3^- -fed plants (data not shown). NO_3^- supplied tea plants contained larger concentrations of organic acids than those receiving NH_4^+ , which is interpreted as a result for charge compensation and maintenance of intracellular pH stability (Marschner, 1995). This also provides an explanation for the effect of high root-zone pH, of which substantial accumulation of organic acids was observed as a result of significantly larger uptake of cations.

Reduced plant growth with NO_3^- compared to NH_4^+ could be due to low NO_3^- absorption rate (Lavoie et al., 1992), inefficient assimilation owing to low nitrate reductase activity (Poonnachat and Darnell, 2004), or be explained by the combination of both factors. Although it is well established that N uptake is

controlled by the demand imposed by growth rate (Touraine et al., 2001), present data apparently indicate that the inefficient absorption of NO_3^- likely played an important role because the low RGR of these plants closely related to their low NO_3^- absorption rate (see Figure 3). The conclusion is further supported by several lines of other evidences. Firstly, the low concentration of total N in mature leaves of NO_3^- -fed plants, which was close to the critical deficiency level around 30 mg g^{-1} (Bonheure and Willson, 1992), indicated these plants possibly suffered from N deficiency. The visual N deficiency-like symptoms (yellowish leaves with low chlorophyll contents) and smaller concentrations of free amino acids were also supporting indications of inadequate N status in these plants. Secondly, tissue N concentration significantly correlated with specific N (NH_4^+ and NO_3^-) absorption rate, suggesting the reduced total N concentration in NO_3^- supplied plants was due to the low NO_3^- absorption rate. Thirdly, only small amounts of NO_3^- accumulated in mature leaves of NO_3^- -fed plants while similar concentrations in the roots of NO_3^- and $\text{NH}_4^+ + \text{NO}_3^-$ supplied plants, regardless of their largely different specific NO_3^- absorption rates, imply that the reduction of NO_3^- might have not been limited. From an evolutionary perspective, the originating center of tea plants is believed to be in the natural forest of southeast China (Tea Research Institute, 1985), where they co-dominate with other dominant species to form climax vegetation (L. Chen, personal communication). The preference for NH_4^+ and the poor utilization capacity for NO_3^- in tea may reflect its ecological position as a late successional species that is adapted to forest soils enriched with NH_4^+ as the predominant inorganic N form (Kronzucker et al., 2003).

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Chapter 2

Effect of root-zone pH, nitrogen form and concentration on accumulation of quality related components in green tea

Abstract

The quality of green tea is greatly influenced by concentrations of free amino acids, catechins and caffeine. The effects of root-zone pH, N form and supply level on accumulation of these compounds in young shoots of tea plants (*Camellia sinensis* (L.) O. Kuntze) were studied. Tea plants were hydroponically cultured with NH_4^+ , NO_3^- and $\text{NH}_4^+\text{NO}_3^-$ at pH 4.0, 5.0 and 6.0 in one experiment or supplied with varying N concentrations (0.75, 2.0 and 4.5 mmol L⁻¹, $\text{NH}_4^+:\text{NO}_3^- = 3:1$) in another experiment. Concentrations of free amino acids were considerably larger in NH_4^+ - than in NO_3^- -fed plants. This was attributed to the much larger absorption of NH_4^+ over NO_3^- . Furthermore, the relative allocation of absorbed N to the major free amino acids (particularly theanine and glutamine) was substantially increased by supplying the plants with NH_4^+ nutrition, suggesting that NH_4^+ was more readily assimilated than NO_3^- into theanine, the predominant and unique amino acid in tea plants. Concentration of caffeine was increased in NH_4^+ - and $\text{NH}_4^+\text{NO}_3^-$ -supplied plants whereas concentrations of catechins were reduced in $\text{NH}_4^+\text{NO}_3^-$ -fed plants. Root-zone pH did not influence concentrations of most free amino acids in young shoots except theanine, which was larger at pH 4.0 irrespective of the N form. This was attributed to an accumulation effect as at low pH, growth was reduced more than N absorption. By raising N concentration in the nutrient solution, N allocation to free amino acids increased, which arose largely from a striking increase of the fraction to arginine while the fraction to theanine was marginally affected under high N supply.

Introduction

According to manufacturing processes, tea may be classified into three main types as green tea (non-fermented), black tea (fully-fermented) and oolong tea (semi-fermented). Quality of these types is greatly influenced by organic and inorganic compositions in harvested young shoots, which function as precursors of, and are changed during processing into, substances determining the flavor quality (Hara et al., 1995). Free amino acids, polyphenols or catechins, and caffeine are particularly important groups of such compounds. Though the concentrations of catechins and caffeine largely affect the quality of black tea (Obanda et al., 1997), quality green tea is characterized by high free amino acids and appropriate concentrations of catechins and caffeine (Chen et al., 1985; Wang et al., 1988). Free amino acids have been recognized as essential constituents of quality green tea and are the principal contributor to freshness and mellowness of the infusion (Chen et al., 1985; Mukai et al., 1992; Wang et al., 1988). Free amino acids also react with soluble sugars and catechins under heated conditions during processing, leading to formation of chemical compounds contributing to the aroma property of green tea (Hara und Kubota, 1982; 1983).

Concentrations of total free amino acid in green tea are generally in the range of

10-50 mg g⁻¹. The predominant free amino acid is L-theanine (N⁵-ethyl-glutamine, Thea), accounting for up to 70% of the total in green teas. So far, Thea has been found uniquely in *Camellia sinensis*, and at low concentrations in a few other species from the *Camellia* genus and in mushroom *Xerocomus badius* (Casimir et al., 1960; Tsushida and Takeo, 1984). The quantities of free amino acids are greatly affected by N supply, increasing substantially by generous N supply exceeding growth demand (Okano et al., 1997). Recent studies demonstrated that extremely large amounts of N fertilizers applied to green tea fields to produce products rich in free amino acids, impose however, high production costs and the danger of environmental hazards (Tachibana et al., 1995; Ruan and Wu, 2004).

Ammonium and nitrate are the most important inorganic N sources in soils, that are readily available to plants. Responses of plants to these two N forms are variable and species dependent (Britto and Kronzucker, 2002). Previous experiments have shown preferential uptake and better growth with NH₄⁺ than with NO₃⁻ in tea plants (Morita et al., 1998; Chapter 1). In addition, chemical composition of plants is greatly affected by N forms supplied. Compared to NO₃⁻-fed plants, NH₄⁺ treated plants usually maintain larger concentrations of free amino acids (Raab and Terry, 1995; Lang and Kaiser, 1994). This can be of special interest with regard to product quality of green tea. Theanine has been reported to synthesize in roots from glutamic acid and ethylamine, catalyzed by L-glutamate : ethylamine ligase (Sasaoka et al., 1965; Takeo 1974). Since NH₄⁺ is assimilated to a large extent in the roots while NO₃⁻ assimilation occurs in both roots and leaves (Marschner, 1995; Morita et al., 1998; Takeo, 1980), it was thus hypothesized that the availabilities of these N sources for synthesis of Thea may be largely different.

The most suitable pH for growth of tea plants is around 5.0 (Othieno, 1992; Chapter 1), but studies have shown there are large areas of tea fields with soil pH below 4.0 in China and Japan, the two main green tea producers (Ma et al., 2000; Tachibana et al. 1995). Since uptake of NO₃⁻ or NH₄⁺ depends to some degree on ambient pH (Chapter 1; Henry and Raper; 1989; Marcus-Wyner, 1983; Vessey et al., 1990), it is thus expected that their absorption and assimilation into quality forming free amino acids of green tea might be differently influenced as external pH changes.

The purpose of this study was to assess how concentration of N supply, N form and root-zone pH affect accumulation of and the N allocation to form free amino acids, in particular Thea. The experiments were conducted in nutrient solution in order that the fibrous roots, where Thea is synthesized (Okano and Omae, 1996), could be completely recovered.

Materials and Methods

Conditions of plant growth

Experiment 1

Three seedlings with 3-4 leaves germinated from seeds (cv. Longjing 43) were transplanted to 0.2 mmol L⁻¹ CaSO₄ solution for five days and nutrient solution thereafter. Strength of the nutrient solution was increased stepwise from 1/3 to 2/5 (week 2 and 3), 1/2 (week 4), 3/4 (week 5 to 12) and finally to full strength. The

solution contained the macronutrients (mmol L⁻¹) N 1.7, P 0.07, K 0.67, Ca 0.53, Mg 0.67 and the micronutrients (μmol l⁻¹) Zn 0.67, Cu 0.13, Mn 1.0, B 7, Mo 0.33, EDTA-Fe 4.2 and Al 70. Nitrogen was supplied as NH₄⁺, NO₃⁻ and 50% NH₄⁺ + 50% NO₃⁻. 3,4-dimethyl pyrazole phosphate (DMPP) at 1% of the total N was included to inhibit any potential nitrification in the nutrient solution. The pH of the nutrient solutions was continuously titrated to 4.0, 5.0 and 6.0 for each N source by using H₂SO₄ and NaOH in pH-stat systems. Initially each pot contained 4 L nutrient solution with 3 seedlings, thinned to 2 plants 12 weeks later. The nutrient solution was replaced every week.

Experiment 2

Each of two 2-year-old rooted cuttings cv. Longjing 43 (fresh weight 133 ± 3 g) pre-cultivated in diluted nutrient solution (1/8 full strength) for one year was transferred to pots with 4 L full strength nutrient solution containing macronutrients (mmol L⁻¹) P 0.1, K 1.0, Ca 0.8, Mg 0.4 and micronutrients (μmol L⁻¹) Mn 1.5, Zn 1.0, Cu 0.2, B 10, Mo 0.5, Fe 6.25 as EDTA salt and Al 50. Three treatments with different N concentrations (mmol L⁻¹) in nutrient solutions were imposed: N₁ 0.75, N₂ 2.0, and N₃ 4.5, provided as NH₄⁺ and NO₃⁻ (NH₄⁺:NO₃⁻=3:1). The pH of the nutrient solution was maintained at 5.0 by the pH-stat system as described above. Nutrient solution was replaced at weekly intervals.

Both experiments were conducted in a naturally lit glasshouse. Experiment 1 was carried out for 20 weeks from May to September. Plants were provided with additional light (lamps SON-T AGRO 400 W, Philips) to ensure minimum light intensity of 220 μmol m⁻² s⁻¹ at canopy level from the beginning until 4 weeks after transplanting. Thereafter solely natural light was used. Experiment 2 was conducted from October to February. Additional light with the same light intensity as above was provided over the entire growing period. Maximum air temperature in the glasshouse was approximately 34 °C daytime and minimum 20 °C at night. Relative humidity was maintained around 70% by a humidifier. Samples of young shoots consisting of one bud with two leaves were regularly collected, quickly frozen in liquid N₂ and freeze dried. At the end of the experiments, samples of fibrous roots were collected and prepared in the same way as the young shoots.

Enzyme assay

Glutamine synthetase (GS, EC 6.3.1.2) in fibrous roots and young fully expanded leaves was extracted with a buffer (pH 7.5) containing 50 mmol L⁻¹ Tris, 5 mmol L⁻¹ EDTA, 5 mmol L⁻¹ dithioerythritol (DTE) at a ratio of 10 ml g⁻¹ FW and 5% (w/v) PVPP using a Potter S homogenizer cooled with ice (Gerendás et al., 1998). Extracts were centrifuged at 12,000 g for 10 min. GS activity was determined by a transaminase assay in mixtures containing 50 mmol L⁻¹ imidazol buffer (pH 7.2), 50 mmol L⁻¹ hydroxylamine, 15 mmol L⁻¹ Na₂HAsO₄, 3 mmol L⁻¹ MnCl₂, 50 mmol L⁻¹ glutamine, and 0.1 mmol L⁻¹ ADP. The reaction was conducted at 30°C for 30 min and terminated by addition of 1 ml FeCl₃ reagent. After the mixture was centrifuged, absorbance was read at 500 nm. One unit of enzyme activity corresponded to the formation of 1 μmol γ-glutamyl hydroxamate per gram fresh material per min.

Determination of free amino acids, caffeine, catechins and total N

Plant samples (60 mg) of finely ground powder were extracted with 3 ml H₂O in a hot water bath (100°C) for 5 min. The extract was used for determining free amino acids, caffeine and catechins. Free amino acids were analysed as o-phthalaldehyde derivatives on a reverse phase C₁₈ column (Hypersil ODS, 3 µm, 250 mm x 4.6 mm) using an automated HPLC system (Gerendás et al., 1998). Standards were prepared from authentic compounds and norvaline was used as an internal standard. To compare the relative allocation of N to the synthesis of individual free amino acids, the quantity percentages of N found in the total free amino acid (Amino-N/TN), Thea (Thea-N/TN), Gln (Gln-N/TN), Arg (Arg-N/TN) and other amino acids (excluding Thea, Gln or Arg, Others-N/TN) accounting for the total tissue N (TN) were calculated. Catechins and caffeine were analyzed by HPLC according to the method of Wang et al. (2003). Concentrations of (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC) and (-)-epicatechin gallate (ECG) were quantified according to the relative response factors (at 210 nm) to standards (+)-catechin and (-)-epicatechin (EC) (Wang et al., 2003). Concentration of total N in plant samples was measured by an elemental analyzer (Carlo Erba, Milano).

Statistics

Data from Experiment 1 were subjected to two-way analysis of variance (ANOVA) to examine the effects of N form, pH and their interaction. One-way ANOVA and LSD test were performed for data from Experiment 2.

Results

Experiment 1

Biomass production of young shoots

Young shoot biomass production was significantly larger at pH 5.0 than pH 6.0 for plants exclusively receiving NH₄⁺ or NO₃⁻ (Table 1). The differences between pH 4.0 and pH 6.0 were insignificant for NH₄⁺-supplied plants but were pronounced for NO₃⁻-fed plants. Plants receiving NH₄⁺+NO₃⁻ did not differ significantly in their biomass production among different root-zone pH treatments. Young shoot growth was significantly increased in plants supplied with NH₄⁺ or NH₄⁺+NO₃⁻ compared to those with NO₃⁻ at pH 4.0 and pH 6.0 and was slightly ($p>0.05$) larger for NH₄⁺- than for NH₄⁺+NO₃⁻-supplied plants (Table 1). Differences among the three N sources were insignificant at pH 5.0 though NH₄⁺- fed plants produced slightly larger yield.

GS enzyme activity

Activity of GS in fibrous roots and young leaves was enhanced in NH₄⁺-fed plants compared to those supplied with NO₃⁻ (Table 1). Roots of NH₄⁺-fed plants always had substantially greater GS activity than those provided with NH₄⁺+NO₃⁻ whereas in young leaves their differences were insignificant. The effect of pH was insignificant in most cases except in young leaves of plants supplied with NO₃⁻, which showed reduced enzyme activity at pH 4.0 compared to those at pH 5.0 or pH 6.0.

Table 1. Yield of young shoots, GS activity in fibrous roots and young leaves of tea plants grown under a regime of different N forms and root-zone pH.

Data are means \pm standard deviations (n = 3-4).

N form	pH	Young shoots (g pot ⁻¹)	GS activity ($\mu\text{mol g}^{-1}$ fw. min ⁻¹)	
			Fibrous roots	Young leaves
NH ₄ ⁺	4.0	1.55 \pm 0.49	7.13 \pm 1.36	77.0 \pm 4.8
	5.0	2.27 \pm 0.26	7.35 \pm 1.76	84.1 \pm 10.1
	6.0	1.60 \pm 0.22	7.94 \pm 2.46	93.5 \pm 10.7
NO ₃ ⁻	4.0	0.77 \pm 0.38	0.55 \pm 0.63	53.1 \pm 4.3
	5.0	1.80 \pm 0.21	0.39 \pm 0.54	69.4 \pm 7.5
	6.0	0.30 \pm 0.16	0.52 \pm 0.47	66.6 \pm 3.9
NH ₄ ⁺ +NO ₃ ⁻	4.0	1.36 \pm 0.35	1.89 \pm 1.15	87.1 \pm 4.5
	5.0	1.68 \pm 0.51	2.72 \pm 1.27	74.1 \pm 16.4
	6.0	1.46 \pm 0.25	3.29 \pm 2.43	80.9 \pm 13.7
ANOVA (<i>F</i> value)				
<i>Factor</i>	N form	19.79**	46.18**	14.86**
	pH	20.02**	0.47	1.81
	N form \times pH	3.82*	0.16	2.79*

*: $p < 0.05$; **: $p < 0.01$.

Free amino acids

Theanine, Gln, Glu and Arg were the most abundant free amino acids (amides), comprising 82 to 92% of the total pool in young shoots (Table 2). Total free amino acid was substantially increased in NH₄⁺-supplied plants. In particular, concentrations of Thea, Gln and Arg were 3-9, 4-7 and 2-21 fold greater in NH₄⁺- than in NO₃⁻-fed plants, respectively. Young shoots of NH₄⁺+NO₃⁻-supplied plants usually contained intermediate levels of amino acids. Similarly, the concentrations of total free amino acids in the fibrous roots of NH₄⁺-fed plants were 4 - 17 fold greater than those of NO₃⁻ treated plants (Figure 1). The most abundant and considerably affected amino acids were Thea, Gln, and Arg, which were 3-25, 16-44, and 11-150 fold greater in NH₄⁺- than in NO₃⁻-supplied plants, respectively. Glutamate, Asp and other amino acids followed similar trends but to a lesser extent. In NH₄⁺+NO₃⁻-fed roots, concentrations of most amino acids were at intermediate levels between those of the NH₄⁺- and NO₃⁻-treatments. There was a significant correlation between the concentration of Thea in young shoots and roots (Figure 2A).

The proportional distribution of total N among various N fractions (expressed as N-fraction/total N) in fibrous roots and young shoots was calculated. Fractions analyzed were total free amino acids (Amino-N/TN), Thea (Thea-N/TN), Gln (Gln-N/TN) and other amino acids (Others-N/TN, excluding Thea and Gln) (Table 3). Amino-N accounted for almost half of the total N (Amino-N/TN from 43.4 to 52.1%) in roots of NH₄⁺-supplied plants, which were 3-10 fold greater than NO₃⁻-treated roots. This was reflected by the considerably larger fractions of Thea-N/TN and Gln-N/TN. Amino-N/TN ratios in roots of NH₄⁺-supplied plants were nearly double those in roots receiving NH₄⁺+NO₃⁻ owing to the markedly different Gln-N/TN while the Thea-N/TN ratios were at similar levels. Differences in Amino-N and Amino-N/TN between NH₄⁺+NO₃⁻ and NO₃⁻ treatments could be largely attributed to the fraction of Thea-N/TN and only marginally to the others (Others-N/TN). The

Amino-N/TN, Thea-N/TN, Gln-N/TN and Others-N/TN ratios were much smaller in young shoots than in roots while their differences between N forms followed a similar trend as in the roots (Table 3).

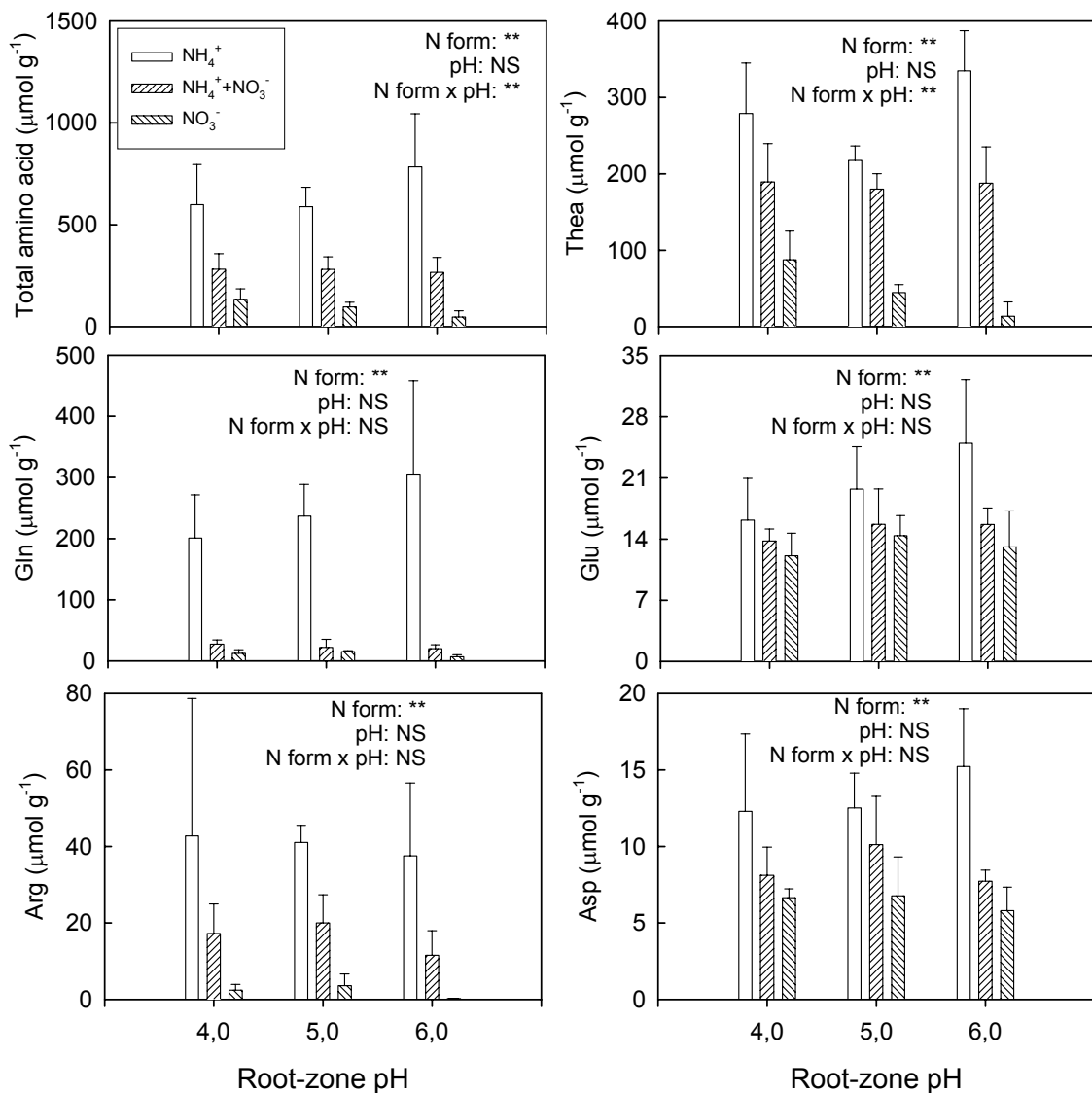


Figure 1. Concentrations of total and major free amino acids in fibrous roots of tea plants grown under a regime of different N forms and root-zone pH. Bars are standard deviations of four replications. Results of two-way ANOVA analysis are indicated.

** : $p < 0.01$, * : $p < 0.05$, NS: not significant.

Concentrations of total free amino acid and particularly Thea in young shoots were higher at pH 4.0 than at pH 5.0 or pH 6.0, whereas most other free amino acids were not influenced by pH (Table 2). Concentrations of Thea (Figure 1), Ala, GABA, and Orn (data not shown) in roots were also affected by pH treatment or by N form and pH interaction. When supplied with NH₄⁺, Thea concentrations in roots were larger at pH 4.0 or 6.0 than at pH 5.0, whereas they were higher at pH 4.0 than at pH 5.0 or pH 6.0 when supplied with NO₃⁻. Allocation of N to free amino acids (Amino-N, Amino-N/TN and Thea-N/TN) was affected by pH only when NO₃⁻ served as the sole N source (Table 3).

Table 2. Free amino acid concentrations ($\mu\text{mol g}^{-1}$) in young shoots of tea plants grown under a regime of different N forms and root-zone pH.
Data are means (n=4)

Amino acid	pH 4.0			pH 5.0			pH 6.0			ANOVA test		
	NH_4^+	NO_3^-	$\text{NH}_4^++\text{NO}_3^-$	NH_4^+	NO_3^-	$\text{NH}_4^++\text{NO}_3^-$	NH_4^+	NO_3^-	$\text{NH}_4^++\text{NO}_3^-$	N form	pH	N x pH
Thea	87.31	22.63	47.69	41.01	13.87	31.47	45.18	5.18	33.83	**	**	NS
Gln	59.00	8.63	38.55	46.13	10.77	23.85	43.33	7.76	27.88	**	NS	NS
Glu	29.06	15.15	26.08	23.72	15.32	21.82	27.00	20.13	23.10	**	NS	NS
Arg	24.80	1.19	2.86	8.57	0.87	2.20	5.30	2.98	1.77	**	NS	NS
Ala	2.69	3.47	3.50	3.06	2.80	2.38	2.69	3.09	2.10	NS	NS	NS
Asn	0.86	0.36	0.87	0.70	0.43	0.68	0.66	0.41	0.73	**	NS	NS
Asp	8.78	4.44	8.04	7.47	4.53	8.29	8.94	5.37	7.64	**	NS	NS
GABA	1.05	0.49	1.16	1.12	0.40	0.87	1.00	0.35	0.57	**	*	NS
Gly	1.52	0.90	0.85	1.22	0.85	0.72	1.66	0.76	1.14	**	NS	NS
His	2.05	0.32	5.94	1.04	0.32	0.47	0.74	0.31	0.57	NS	NS	NS
Ile	0.28	0.22	0.22	0.25	0.20	0.19	0.29	0.20	0.19	*	NS	NS
Leu	0.44	n.d.	0.18	0.29	n.d.	0.18	0.36	n.d.	0.16	**	NS	NS
Lys	1.51	0.17	n.d.	0.50	0.15	n.d.	0.43	0.17	n.d.	**	NS	NS
Met	0.05	0.09	0.08	0.11	0.09	0.01	n.d.	0.06	0.08	NS	NS	NS
Phe	0.56	0.37	0.51	0.52	0.35	0.44	0.59	0.33	0.42	**	NS	NS
Ser	2.44	1.75	3.32	3.38	1.97	3.09	3.98	1.66	3.06	**	NS	NS
Thr	1.45	0.63	1.18	1.56	0.67	1.09	1.28	0.71	0.99	**	NS	NS
Try	0.43	0.20	0.35	0.48	0.25	0.34	0.39	0.20	0.33	**	NS	NS
Tyr	1.79	0.30	0.66	1.42	0.27	0.52	0.96	0.26	0.49	**	*	NS
Val	0.58	0.32	0.48	0.79	0.29	0.40	0.96	0.32	0.32	**	NS	*
Sum	226.65	61.61	142.50	143.33	54.39	99.02	145.73	50.42	105.35	**	*	NS

n.d.: not detected; *: $p < 0.05$; **: $p < 0.01$; NS: not significant

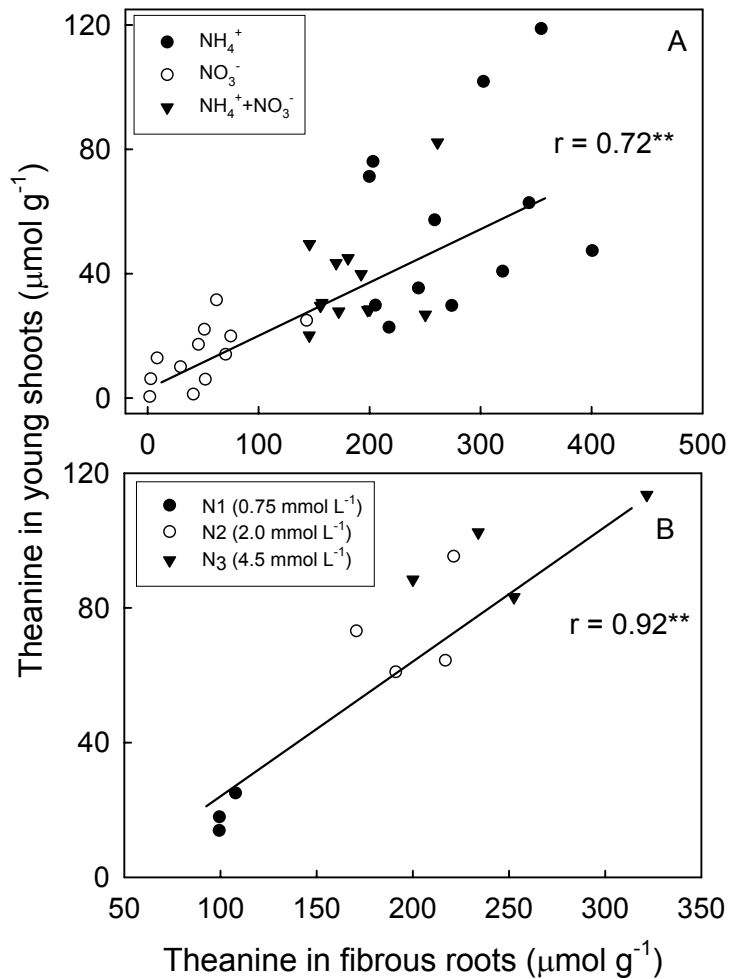


Figure 2. Correlation between concentrations of theanine in fibrous roots and in young shoots of tea plants grown under a regime of different N forms and root-zone pH (A) or varying external N supplies (B). **: $p < 0.01$.

Caffeine and catechins

Concentration of caffeine was higher in plants supplied with NH_4^+ or $\text{NH}_4^+ + \text{NO}_3^-$ than in those given NO_3^- . Increasing pH caused in general, caffeine concentration decline, being most pronounced in NH_4^+ - or NO_3^- -treated plants (Table 4). The most abundant catechins in young shoots were (-)-EGCG and (-)-EGC, which were reduced in $\text{NH}_4^+ + \text{NO}_3^-$ - compared to NH_4^+ - or NO_3^- -supplied plants (Table 4). Only EGC concentration responded with a significant decline when root-zone pH increased to 6.0. Concentrations of (-)-EGC and (-)-EC were unaffected by N forms and pH levels.

Experiment 2

Concentrations of total free amino acid, Thea and others (Gln, Glu, Arg, Asp) in young shoots were significantly raised by increasing N supplies, being 4, 5, 7, 3, 21 and 3 fold larger in N_3 than in N_1 treatment, respectively (Table 5). Similarly, with increasing N concentration in the nutrient solution more free amino acids accumulated in fibrous roots (Figure 3). The proportion of individual amino acids in the total pool varied differently in response to changes of external N concentration.

In young shoots, concentration percentage of Thea to total free amino acid increased from 41.5% at N₁ to 48% at N₂ and remained unchanged at N₃ (47.6%). In roots, the percentage of Thea decreased from 82% at N₁ to 74% at N₂ and further to 67% at N₃. The percentages of Gln and Arg in both young shoots and roots increased while those of Glu and other amino acids decreased constantly with increasing N supply. Correspondingly, the ratios of Thea-N/TN in roots and young shoots (data not shown) increased only slightly ($p>0.05$) at N₂ and remained unchanged at N₃ (Figure 4). On the other hand, the Arg-N/TN ratio increased constantly with increasing external N concentrations. A significant positive correlation between Thea in young shoots and in roots was once again observed (Figure 2B). Concentrations of catechins in young shoots significantly decreased in N₂ and N₃ compared to N₁ treatment while caffeine increased with increasing N supply (data not shown).

Table 3. Total tissue N content (TN), total free amino acids-N (Amino-N) and proportional distribution of total N among N-fractions in roots and young shoots of plants as affected by N forms and root-zone pH. Data are means \pm standard deviations ($n = 4$).

N form	pH	Total N (TN) (mg g ⁻¹)	Amino-N (mg g ⁻¹)	Amino- N/TN (%)	Thea- N/TN (%)	Gln-N/TN (%)	Others- N/TN (%)
<i>Root</i>							
NH ₄ ⁺	4.0	39.7 \pm 7.3	17.4 \pm 5.1	43.3 \pm 4.7	19.6 \pm 2.0	13.9 \pm 3.1	9.8 \pm 3.5
	5.0	35.3 \pm 2.4	17.0 \pm 1.1	48.3 \pm 2.0	17.4 \pm 2.2	18.7 \pm 2.9	12.3 \pm 1.4
	6.0	42.5 \pm 2.3	22.2 \pm 3.6	52.1 \pm 7.0	22.0 \pm 2.8	20.1 \pm 10.1	9.9 \pm 2.7
NO ₃ ⁻	4.0	23.2 \pm 1.5	3.5 \pm 1.0	15.0 \pm 3.7	10.5 \pm 4.2	1.5 \pm 0.7	3.0 \pm 0.5
	5.0	22.0 \pm 1.5	2.4 \pm 0.4	11.1 \pm 1.9	5.7 \pm 1.3	1.9 \pm 0.3	3.5 \pm 1.0
	6.0	19.1 \pm 3.1	1.0 \pm 0.6	5.0 \pm 2.3	1.8 \pm 2.2	1.0 \pm 0.5	2.2 \pm 0.2
NH ₄ ⁺ +NO ₃ ⁻	4.0	30.1 \pm 1.4	8.0 \pm 0.8	26.5 \pm 2.8	17.6 \pm 4.4	2.6 \pm 0.7	6.3 \pm 1.7
	5.0	30.2 \pm 1.5	7.9 \pm 1.0	26.0 \pm 2.0	16.7 \pm 1.1	2.0 \pm 1.1	7.3 \pm 1.3
	6.0	29.5 \pm 1.7	7.3 \pm 1.7	24.7 \pm 4.5	17.7 \pm 3.6	1.9 \pm 0.6	5.1 \pm 1.9
ANOVA (<i>F</i> value)							
<i>Factor</i>	N form	98.64**	167.61**	298.17**	77.41**	73.46**	53.53**
	pH	1.13	0.68	0.36	2.88	0.79	3.44
N form \times pH		3.19*	3.60*	6.19**	4.53**	1.22	0.35
<i>Shoot</i>							
NH ₄ ⁺	4.0	49.4 \pm 5.9	6.4 \pm 2.4	12.7 \pm 3.9	4.9 \pm 1.2	3.3 \pm 0.9	4.5 \pm 2.7
	5.0	46.5 \pm 3.4	3.6 \pm 1.6	8.0 \pm 4.2	2.6 \pm 1.7	2.9 \pm 1.9	2.6 \pm 0.6
	6.0	42.2 \pm 3.5	3.5 \pm 0.6	8.4 \pm 0.9	3.0 \pm 0.8	2.9 \pm 0.4	2.5 \pm 0.1
NO ₃ ⁻	4.0	33.9 \pm 0.3	1.4 \pm 0.3	4.0 \pm 0.9	1.9 \pm 0.6	0.7 \pm 0.2	1.5 \pm 0.3
	5.0	37.1 \pm 3.1	1.2 \pm 0.2	3.1 \pm 0.4	1.0 \pm 0.5	0.8 \pm 0.2	1.3 \pm 0.1
	6.0	35.4 \pm 3.1	1.0 \pm 0.9	2.9 \pm 2.5	0.4 \pm 0.5	0.6 \pm 0.6	1.9 \pm 1.4
NH ₄ ⁺ +NO ₃ ⁻	4.0	46.4 \pm 7.6	3.5 \pm 1.6	7.3 \pm 2.5	2.7 \pm 1.1	2.2 \pm 1.0	2.3 \pm 0.9
	5.0	46.1 \pm 6.0	2.3 \pm 0.5	4.9 \pm 0.7	1.9 \pm 0.2	1.4 \pm 0.9	1.6 \pm 0.1
	6.0	46.9 \pm 5.5	2.4 \pm 0.8	5.2 \pm 1.2	2.0 \pm 0.5	1.6 \pm 0.6	1.5 \pm 0.2
ANOVA (<i>F</i> value)							
<i>Factor</i>	N form	20.95**	23.58**	22.71**	20.66**	19.37**	8.20**
	pH	0.53	5.52*	4.98*	8.99**	0.67	2.58
N form \times pH		1.15	1.54	0.76	1.32	0.27	1.35

*: $p<0.05$; **: $p<0.01$. NS: not significant

Table 4. Caffeine and catechin concentrations of (mg g^{-1}) in young shoots of tea plants grown under a regime of different N forms and root-zone pH.

Data are means \pm standard deviations ($n = 4$).

Treatment		Caffeine	(-)-EGCG	(-)-EGC	(-)-EC	(-)-ECG
N form	pH					
NH_4^+	4.0	20.1 \pm 3.2	31.0 \pm 5.6	21.3 \pm 4.2	5.2 \pm 1.2	4.1 \pm 1.6
	5.0	19.0 \pm 2.8	33.7 \pm 5.8	22.2 \pm 4.9	3.5 \pm 2.3	3.3 \pm 0.4
	6.0	14.6 \pm 1.5	25.1 \pm 5.0	14.1 \pm 2.5	3.0 \pm 0.8	2.6 \pm 0.7
NO_3^-	4.0	13.2 \pm 0.2	29.9 \pm 5.1	20.9 \pm 7.7	4.2 \pm 1.5	3.2 \pm 1.0
	5.0	15.1 \pm 2.2	35.1 \pm 7.8	23.0 \pm 3.3	4.6 \pm 0.9	3.7 \pm 1.0
	6.0	12.4 \pm 1.2	31.1 \pm 4.0	18.2 \pm 2.8	4.0 \pm 0.8	4.1 \pm 0.5
$\text{NH}_4^++\text{NO}_3^-$	4.0	19.3 \pm 4.1	26.8 \pm 3.1	17.3 \pm 3.0	4.1 \pm 0.5	3.0 \pm 0.6
	5.0	15.9 \pm 1.7	20.8 \pm 2.8	10.9 \pm 1.4	3.0 \pm 0.9	2.4 \pm 0.8
	6.0	16.0 \pm 1.8	26.0 \pm 4.5	12.0 \pm 3.5	3.1 \pm 1.0	3.0 \pm 0.8
ANOVA (<i>F</i> -value)						
<i>Factor</i>	N form	11.28**	6.98**	10.60**	1.66	2.88
	pH	5.87**	0.76	5.01*	2.74	0.31
	N form \times pH	2.13	2.53	1.92	1.18	2.13

*. $p < 0.05$; **. $p < 0.01$.

Discussion

Effect of pH

The effects of N form and root-zone pH on growth of young tea shoots were similar to those for whole plant biomass production discussed elsewhere (Chapter 1), therefore, will not be discussed here. Though plant growth was considerably influenced, concentrations of most catechins, except (-)-EGC, in young shoots were unaffected by external pH treatments. However, concentrations of (-)-EGC and caffeine were generally reduced at pH 6.0, corresponding well to the inferior growth performance at this pH. Concentrations of total free amino acid in young shoots and roots were increased by declining pH, caused by rising Thea concentration as the dominant free amino acid. This was surprising since absorption of both NH_4^+ and NO_3^- declined from pH 5.0 to pH 4.0 (Chapter 1). This is explained by specific absorption rates of NH_4^+ and NO_3^- (per root dry matter) which at pH 4.0 were reduced by 15% and 37%, respectively, whereas at the same time biomass production of young shoots was diminished by 32% for NH_4^+ -treated plants and by 57% for NO_3^- -treated plants. Consequently greater Thea concentration was possibly a result of an accumulation effect when growth of young shoots decreased more than N absorption at pH 4.0.

Effect of N form

Concentration of caffeine in young shoots was increased by NH_4^+ nutrition compared to NO_3^- . This could be explained by difference in the N status (as indicated by concentrations of total N and free amino acids) of plants supplied with these N forms. The reason for reduced concentrations of catechins in young shoots of plants supplied with $\text{NH}_4^++\text{NO}_3^-$ was unclear. In callus culture formation of catechins is

reduced by NH_4^+ but attains the highest concentration when NO_3^- served as the sole N source in the culture medium (Cheng et al., 1994).

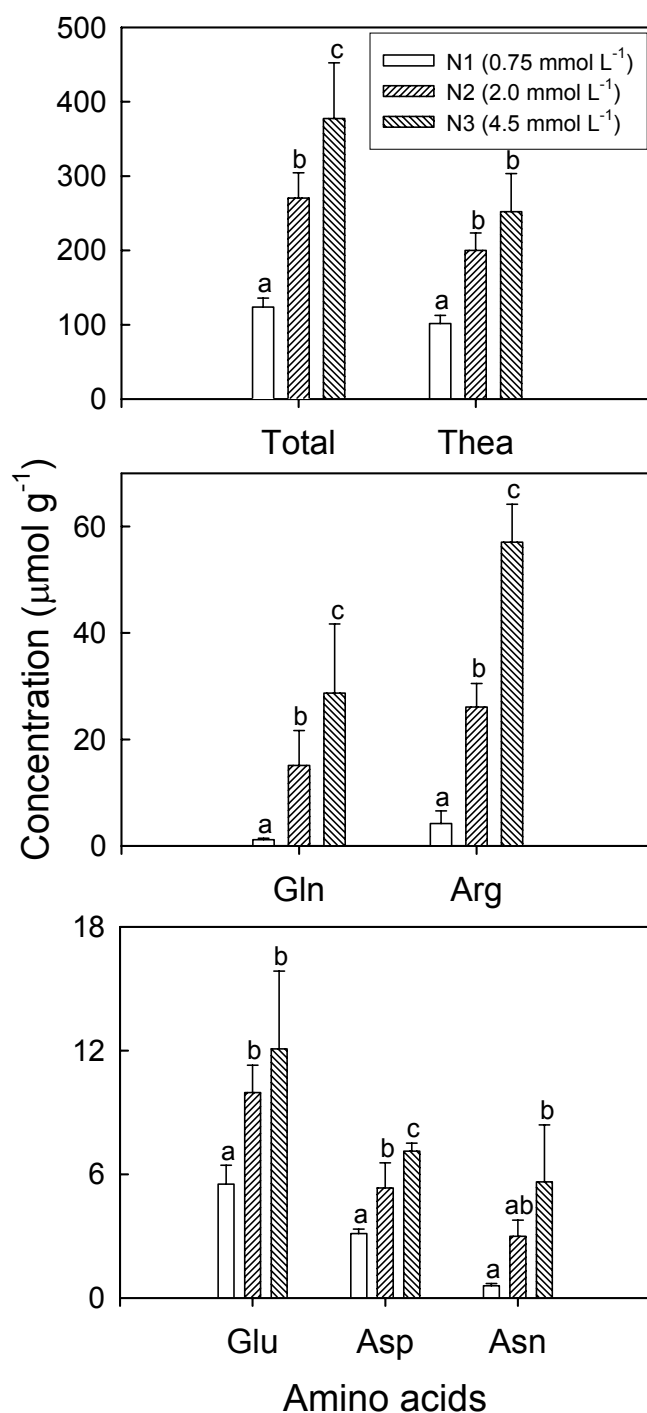


Figure 3. Total and major free amino acid concentrations in fibrous roots of tea plants exposed to varying N supplies. Bars are standard deviations of four replications. Different letters above columns within each amino acid indicate significant ($p < 0.05$) difference by LSD test.

Table 5. Free amino acids ($\mu\text{mol g}^{-1}$) in young shoots of tea plants exposed to varying N supplies. Data are means \pm standard deviations ($n = 3-4$)

Amino acids	N concentration (mmol L^{-1})		
	0.75 (N_1)	2.0 (N_2)	4.5 (N_3)
Thea	18.97 \pm 5.65a	73.47 \pm 15.44b	96.90 \pm 13.77c
Gln	4.68 \pm 1.60a	20.59 \pm 3.24b	31.06 \pm 2.16c
Glu	8.37 \pm 1.67a	18.30 \pm 2.20b	24.93 \pm 2.24c
Arg	1.06 \pm 0.72a	14.72 \pm 5.53b	22.72 \pm 7.51b
Ala	1.03 \pm 0.11a	2.86 \pm 0.23b	3.67 \pm 0.48c
Asn	0.88 \pm 1.11	0.77 \pm 0.13	1.25 \pm 0.26
Asp	2.63 \pm 1.43a	7.10 \pm 0.71b	8.71 \pm 1.05b
GABA	0.29 \pm 0.03a	0.69 \pm 0.20b	0.91 \pm 0.20b
Gly	1.09 \pm 0.24	1.73 \pm 0.83	1.63 \pm 0.52
His	0.29 \pm 0.06	0.55 \pm 0.08	1.06 \pm 0.68
Ile	1.33 \pm 0.43	2.04 \pm 1.55	0.87 \pm 0.49
Met	0.10 \pm 0.03	0.19 \pm 0.09	0.18 \pm 0.03
Phe	0.70 \pm 0.47	0.91 \pm 0.47	0.67 \pm 0.11
Ser	1.72 \pm 0.49a	3.79 \pm 0.29b	5.05 \pm 0.25c
Thr	0.60 \pm 0.15a	1.28 \pm 0.09b	1.58 \pm 0.17c
Try	0.47 \pm 0.29	0.81 \pm 0.43	0.74 \pm 0.04
Tyr	1.10 \pm 0.12	1.75 \pm 0.60	1.11 \pm 0.11
Val	0.40 \pm 0.27	0.68 \pm 0.06	0.58 \pm 0.03
Sum	45.69 \pm 8.55a	152.22 \pm 27.13b	203.58 \pm 20.51c

Different letters following data on the same line indicate significant ($p < 0.05$) difference by LSD test.

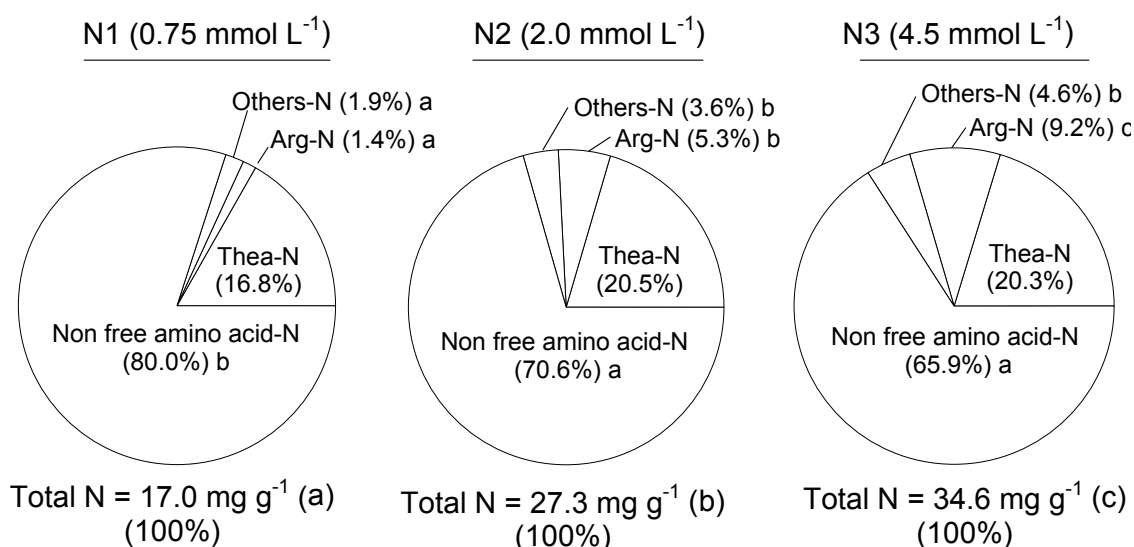


Figure 4. Proportional distribution of total N among N-fractions of theanine (Thea-N), arginine (Arg-N), other free amino acids excluding Thea and Arg (Others-N), and compounds not belonging to free amino acids (Non amino acid-N) in fibrous roots of tea plants exposed to varying N supplies. Data are means of four replications. Different letters following data within the same fractions indicate significant ($p < 0.05$) differences by LSD test.

Considerably larger concentrations of total N and free amino acids in roots of NH_4^+ - supplied plants than in NO_3^- -fed plants were observed. This might be attributed to the much larger specific absorption rate (per root dry matter) of NH_4^+ over NO_3^- when individually supplied (Chapter 1). Moreover, the relative allocation of absorbed N to free amino acids was markedly different for NH_4^+ - and NO_3^- -fed plants. Thea-N/TN and Gln-N/TN ratios were much greater in NH_4^+ treated plants, indicating that more N was assigned to Thea and Gln, than in plants receiving NO_3^- . The Thea/TN ratios were similar between $\text{NH}_4^++\text{NO}_3^-$ and NH_4^+ treatments, regardless of their different total N concentrations in plant tissues. These results suggest synthesis of Thea was largely affected by external N forms and that NH_4^+ was more readily incorporated into Thea than NO_3^- . Such a difference might arise from the effect of N form on enzyme activity, the compartmentation of the enzymes and intermediates of their assimilations. The absorbed NH_4^+ is probably assimilated predominantly in roots via GS-GOGAT pathway whereas NO_3^- is reduced to NH_4^+ and incorporated into amino acids, most likely in either roots or leaves. In the present study, GS activity increased by raising the NH_4^+ level (comparing NO_3^- , $\text{NH}_4^++\text{NO}_3^-$ and NH_4^+ treatments). This explains the changes of Gln concentrations and Gln-N/TN in plants. Since synthesis of Thea occurs in roots (Sasaoka et al., 1965; Takeo 1974), the substrates for Thea synthesis might be readily available and maintained at higher levels in root cells of NH_4^+ supplied plants. The linear positive correlation between Thea in roots and young shoots suggests that its concentration in young shoots depends on its synthesis in and subsequent translocation from roots. In the experiment, relatively large amounts of Thea in fibrous roots, without any harm to plant growth, was observed. This contradicts the viewpoint that high concentration of Thea was toxic to tea plants (Choudhury et al., 1983), and suggests that high Thea concentrations in roots are even a prerequisite for high concentrations in young shoots, hence for high quality green tea.

Effect of N supply concentration

Though increasing N supply from N_1 to N_2 caused an increase in biomass production of young shoots (data not shown), a further increase in N concentration did not lead to further biomass growth. This suggests N_2 (2 mol L^{-1}) might have already been sufficient for growth. However, a distinct accumulation of free amino acids in young shoots occurred when N was supplied at the N_3 level, exceeding growth demand. The tea specific non-protein amino acid Thea was the predominant free amino acid in all three N treatments. Its proportion in the total free amino acid of young shoots increased only when external N supplies rose from N_1 to N_2 and changed little when the N level was raised to N_3 . In roots the ratio of Thea to total free amino acid decreased continuously from N_1 to N_3 though its absolute concentration was still increasing. This was very likely a result of dilution effect arising from the drastic increase of other free amino acids, particularly Arg and Gln. The observation became more apparent by comparing the ratios of N in individual free amino acids to total N, especially in fibrous roots in the case of NH_4^+ assimilation and Thea synthesis (see Figure 4). These results suggest that under high external N supply, absorbed N had been increasingly used for synthesis of Gln and

more strikingly for Arg. This finding is in accordance with previous observations showing Arg extraordinarily accumulated in tea shoots under extremely high N supplies (Okano et al., 1997) or in other plant species (Näsholm and Ericsson, 1990; Warren and Adams, 2000). The reason for accumulation of Arg in tea is unknown but has been considered as an indication of disorder in the N metabolism of tea plants (Okano et al., 1997).

To produce quality green tea with elevated concentrations of free amino acids, N fertilizers have been applied in extremely large quantities. In some Japanese green tea fields up to 1470 kg N ha⁻¹ year⁻¹ on average was applied (Tachibana et al., 1995). In Zhejiang province of China, N applied in green tea fields ranged from 279 to 2104 kg ha⁻¹ year⁻¹ (Ruan and Wu, 2004). Such huge N inputs increase production costs and impose the danger of environmental hazards. The present experiments clearly indicated that excessively absorbed N under condition of high N supply was in a relative sense, increasingly used for synthesis of arginine, not for theanine. Furthermore, due to nitrification even in strongly acidic tea soils, high NO₃⁻ levels were found in tea fields receiving large amounts of N (Hayatsu and Kosuge 1993; Kihou and Yuita, 1991), although N fertilizers have been supplied in the form of NH₄⁺ (e.g. as urea). The present results indicate that NO₃⁻ was only marginally effective in promoting accumulation of free amino acids particularly Thea. This may help to explain the reason for applying such large amounts of N aiming for improved green tea quality. Only the supply of NH₄⁺ led to larger pools of free amino acids, especially Thea, and caffeine. These results therefore, suggest a promising N management of maintaining high levels of NH₄⁺, for example by applying nitrification inhibitors, in green tea plantations.

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Chapter 3

Concentrations of primary and secondary metabolites related to quality of tea plants (*Camellia sinensis* (L.) O. Kuntze) in response to nitrogen supply

Abstract

Quality of tea depends on abundance of catechins, free amino acids and caffeine in young shoots. The present experiment investigated the effect of N concentrations in nutrient solution (N_1 0.35, N_2 0.75, N_3 1.5 and N_4 4.5 mmol L⁻¹) on accumulation of these quality related components in tea plants. Concentrations of major catechins (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) in young shoots were reduced only by the highest N supply, while the predominant catechin (-)-epigallocatechin gallate (EGCG) was unaffected. Biosynthesis of catechins did not appear to be related to concentration of free phenylalanine and activity of phenylalanine-ammonia lyase. Concentrations of glucose and fructose in both young shoots (sink) and recently matured leaves (source) significantly decreased in plants receiving the highest N concentration whereas total N, free amino acids, and caffeine concentrations as well as activity of phosphoenolpyruvate carboxylase and glutamine synthetase increased with increasing external N supply. These results suggest with increasing N supply more carbon resources were allocated to N metabolism, leading to decreased substrate available for formation of catechins in young shoots. Increasing N concentration in the nutrient solution generally promoted accumulation of free amino acids. However, the ratio of Thea to total free amino acid augmented when external N supply rose from N_1 to N_3 but decreased with further increase of N supply due to a dilution effect. The ratios of Arg and Gln to total free amino acid increased sharply only with the highest N supply, indicating that relatively more absorbed N was allocated to synthesis of these amino acids. This is possibly caused by reduced carbohydrate concentration, thus, an imbalanced C : N status in roots, which induced synthesis of amino acids with low C : N ratios to improve carbon economy.

Introduction

Phenolic compounds are a large family of secondary metabolites found in plant tissues. Flavonoids, the main phenolic compounds, comprise 20-40% of dry matter in young shoots of cultivated tea plants. They are manufactured into products belonging to one of the world's most popular beverages. The predominant flavonoids in tea are catechins (flavan-3-ol): (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC), generally accounting for 10-30% of dry weight. The characteristics of made tea, including color, taste and aroma are directly or indirectly associated with these catechins (Wang et al., 2000). During black tea production, catechins are fully oxidized, catalyzed by polyphenol oxidase, to form two pigment groups, theaflavins (TFs) and thearubigins (TRs), responsible for the typical color and flavor of black tea. Previous experiments have shown significant contribution of TFs and TRs to the quality of black tea (Biswas et al., 1973; McDowell et al., 1995). Consequently, concentrations of catechins, the precursor substances for TFs and TRs, in fresh young

shoots positively correlate with black tea quality (Hilton and Palmer-Jones, 1973; Biswas et al., 1973; Obanda et al., 1997).

Green tea is another important type of beverage currently enjoying increasing popularity worldwide. During the green tea manufacturing process, most catechins are preserved owing to inactivation of enzyme by dry heating or steaming at the initial step and are responsible for bitter and astringent tastes of green tea infusion (Wang et al., 2000). In contrast to black tea, high concentrations of polyphenols or catechins, which tend to make infusion strongly bitter, astringent, and reduce mellowness, are not necessarily required for quality green tea. Mellowness together with freshness of infusion is the dominant characteristics of quality green tea and is contributed principally from free amino acids. Previous experiments have shown green tea quality closely correlates with concentration of free amino acids (Chen et al., 1985; Mukai et al., 1992; Wang et al., 1988). In green tea products the predominant free amino acid is a unique non-protein amide N⁵-ethyl-glutamine (theanine or Thea), accounting for up to 70% of the total free amino acid.

Free amino acids, caffeine and flavonoids are produced from primary or secondary metabolisms. These metabolisms in plants often compete for common substrates (e.g. carbon skeletons); therefore appear to be strictly regulated and coordinated temporally and spatially (Matt et al., 2002; Winkel-Shirley, 2001). Furthermore, they are affected by variable environmental conditions including nutrient availability, light intensity, CO₂ concentration, temperature and biotic stresses (Dixon and Paiva, 1995; Koricheva et al., 1998). In a conceptual model Herms and Mattson (1992) proposed that increasing resource availability reduces the proportion of secondary metabolites along with an enhanced primary production. On the other hand, under resource-limiting conditions, such as nutrient deficiency, carbohydrates, which accumulate in excess of demand by growth, will be allocated to carbon-based secondary metabolism (Bryant et al., 1983). This leads to an increasing accumulation of phenolic compounds.

Nitrogen fertilization varies from 300 to more than 2000 kg ha⁻¹ in tea fields (Owuor, 2001; Ruan and Wu, 2004). However, the effect of N fertilization on tea quality is variable and dependent upon types of tea produced. Experiments investigating the effect of N fertilizers on catechins, and their oxidation derivatives theaflavins and thearubigins in black tea showed inconsistent results. Nitrogen fertilization depressed (-)-EGC and (-)-EC in young shoots whereas it either increased or decreased (-)-EGCG concentration (Hilton et al., 1973). In contrast, recent findings by Venkatesan and Ganapathy (2004) showed total polyphenol concentration is increased by N fertilization. In other experiments, it decreased (Cloughley, 1983; Hilton et al., 1973; Owuor and Odhiambo, 1994), had no clear change (Owuor et al., 1991; 2000) or even increased (Owuor et al., 1987) theaflavins and thearubigins. With regard to green tea, it is generally accepted that N application tends to improve its quality by elevating concentrations of free amino acids. Individual amino acids have their own specific tastes individually or in combination as sweet, salty, sour, bitter and umami (the Japanese word 'umami' means delicious and represents the characteristic sensory property of monosodium glutamate). For instance, Thea has sweet and umami tastes while glutamate (Glu) and aspartate (Asp) have tastes of sour and umami. Therefore, the taste of green tea infusion is not only

affected by concentration of total free acid but, possibly more importantly, by the relative proportion of individual free amino acids. However, there is no clear indication of how synthesis of individual free amino acids, in particular Thea, is affected by external N supply.

The objectives of this experiment were to investigate the effect of N supply level on primary and secondary metabolisms and concentrations of their metabolites in relation with accumulation of major quality-related components in tea plants.

Materials and Methods

Plant cultivation and sampling

Two two-year-old rooted cuttings (fresh weight 107 ± 2 g) pre-cultivated in diluted nutrient solution were transferred to pots with 4 L nutrient solution containing macronutrients (mmol L^{-1}) P 0.1, K 1.0, Ca 0.8, Mg 0.4 and micronutrients ($\mu\text{mol L}^{-1}$) EDTA-Fe 6.3, Mn 1.5, Zn 1.0, Cu 0.2, B 10, Mo 0.5 and Al 50. Four treatments with different concentrations (mmol L^{-1}) of N were added: N₁ 0.3, N₂ 0.75, N₃ 1.5 and N₄ 4.5 provided as NH_4^+ and NO_3^- ($\text{NH}_4^+:\text{NO}_3^-=3:1$) starting from week 1 after transplanting. The pH of the nutrient solution was maintained at 5.0 by a pH stat system. Nutrient solution was replaced weekly. Plants were placed in a naturally lit glasshouse provided with additional artificial light to ensure a minimum light intensity of $220 \mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level. The maximum air temperature during the experimental period was approximately 34°C (daytime) and minimum 22°C (night). Relative humidity was maintained at around 70% by a humidifier.

Samples of young shoots of one bud with two leaves were collected, frozen quickly in liquid N_2 and stored at -18°C until freeze dried. The first young shoot samples were collected at week 8 and continued thereafter when young shoots reached the required stage at intervals of 5-8 days. Samples from the same pot were combined for analyses. The whole plant was destructively sampled at week 24. Recently matured leaves and fibrous roots were frozen quickly in liquid N_2 and freeze dried.

Enzyme activity assay

Young expanding leaves (third leaf below the bud) were taken for enzyme activity assay. After collection leaves were frozen in liquid N_2 until extraction (within 4 hrs). Glutamine synthetase (GS, EC 6.3.1.2) was extracted and determined by transferase activity as previously described (Chapter 2). One unit of GS activity was expressed as the formation of $1 \mu\text{mol } \gamma\text{-glutamyl hydroxamate}$ per gram fresh tissue per min. L-phenylalanine ammonia lyase (PAL, EC 4.3.1.5) was assayed as described by Edwards and Kessmann (1992) and one unit of enzyme activity was expressed as the change of 0.01 unit absorbance per gram fresh tissue per min. Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was measured according to the method of Foyer et al. (1994). One unit of PEPC activity corresponded to $1 \mu\text{mol}$ of NADH oxidized per min.

Nutrient concentration

Plant samples were ashed at 500°C and analyzed for P, K, Mg, Ca, and Mn using an Induced Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Model IRIS-AP, Thermo Jarrel Ash Corp., USA). Total N and C were analyzed in an elemental analyzer (Carlo Erba, Milano).

Measurement of free amino acids, soluble sugars and phenols

Free amino acids and sugars in plant samples were extracted with chloroform : methanol (3 : 7, v/v) in ice for 30 min (Lohaus et al., 2000). Homogenate was then extracted twice by 3 ml of distilled water, evaporated to dry in a rotatory evaporator and re-dissolved in 2 ml ultra pure H₂O. Amino acids were analyzed as o-phthalaldehyde derivatives on a reversed C₁₈ column (Hypersil ODS, 3 µm, 250 mm x 4.6 mm) using an automated HPLC system (Gerendás et al., 1998). Norvaline was used as an internal standard. In the Figures (3, 4 B-F), graphical vector analysis plots were prepared by plotting the ratio (percentage) of individual amino acid to the sum of all free amino acids detected by HPLC (y-axis) against its absolute concentration (x-axis). Concentrations of soluble reduced sugars were measured by ion chromatography (DX 300, Dionex, Idstein, Germany).

Phenols and caffeine were extracted twice with 70% aqueous methanol (v/v) each at 70 °C for 10 min at a ratio of 1:25 (w/v) (Astill et al., 2001). Concentration of total phenol was measured by the Folin-Ciocalteu method using gallic acid as a standard. Catechins and caffeine were analyzed by HPLC on a column packed with ODS-5 ST (5 µm, 150 x 4.6 mm, Grom, Germany). The elution solutions and gradients were essentially the same as previously described (Wang et al., 2003). Standards of catechins and caffeine were prepared from authentic compounds.

Results

Plant growth

Plants supplied with the two lowest N levels (N₁ and N₂) showed typical N deficiency symptoms characterized by chlorotic leaves containing significantly less chlorophyll (data not shown). During later growth stages, plants supplied with N₄ showed marginally scorched leaves resembling K deficiency symptoms, later confirmed by plant analysis. Biomass production of the whole plant was significantly raised by increasing N concentration in the nutrient solution (Table 1). Root growth was unaffected, leading to decrease of root fraction (root : whole plant ratio) with increasing N supply. Improving N supply raised yield of young shoots (one bud and two leaves) by increasing number of young shoots per plant but not the weight per shoot (Table 1).

Enzyme activity

Activity of leaf GS, PEPC and PAL increased with external N concentration (Figure 1). Though there was a clear trend for largest activity in N₄, only the differences between N₃ and N₄ on one hand and N₁ and N₂ on the other, were statistically significant.

Table 1 Biomass production of tea plants as affected by a varied nitrogen supply in the nutrient solution. Data are means \pm standard deviations (n = 4)

N supply (mmol L ⁻¹)	Whole plant (g pot ⁻¹)	Root (g pot ⁻¹)	Root fraction	Young shoots (g pot ⁻¹)	Number of young shoots per pot
0.30 (N ₁)	56.08 \pm 2.89a	26.21 \pm 1.16	0.47 \pm 0.03d	1.00 \pm 0.11a	11.8 \pm 1.9a
0.75 (N ₂)	69.07 \pm 3.18b	28.52 \pm 1.22	0.41 \pm 0.02c	1.79 \pm 0.36b	20.0 \pm 5.1b
1.50 (N ₃)	80.68 \pm 2.44c	28.56 \pm 1.35	0.35 \pm 0.02b	2.55 \pm 0.79b	29.5 \pm 11.2b
4.50 (N ₄)	86.17 \pm 11.12c	25.66 \pm 2.62	0.30 \pm 0.02a	4.18 \pm 0.45c	51.0 \pm 6.6c

Different letters following the data within column indicate significant (p<0.05) difference by LSD.

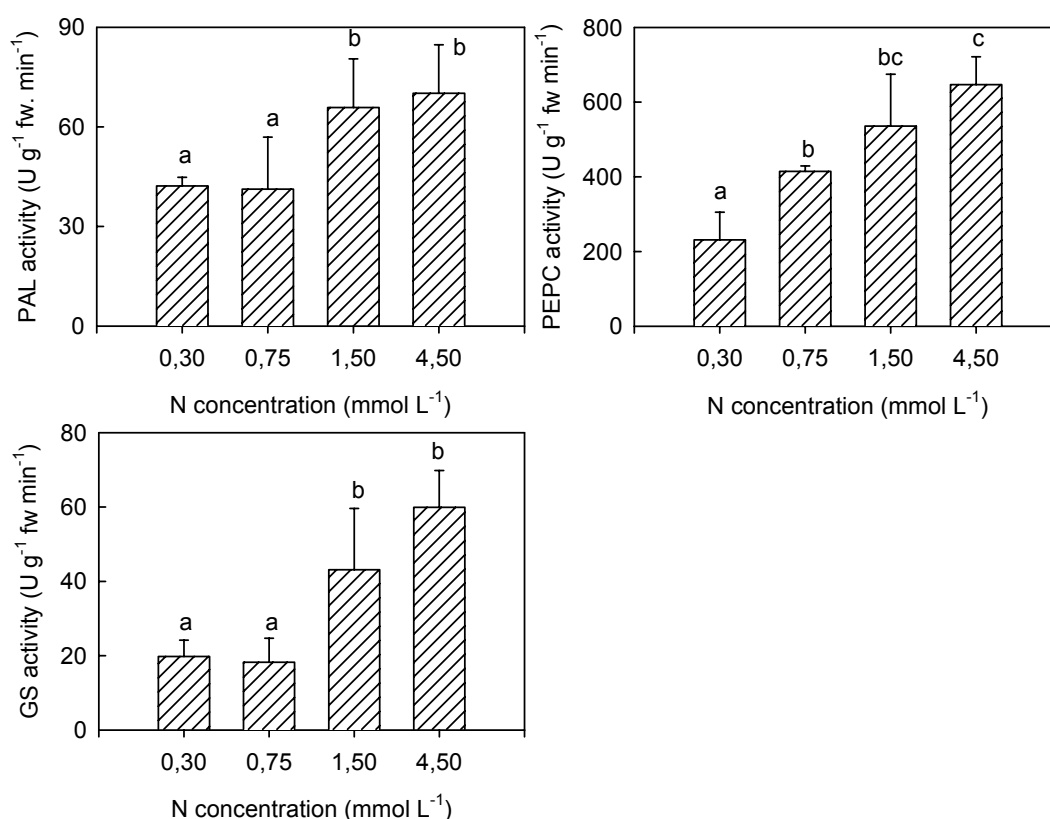


Figure 1 Activity of phenylalanine ammonia-lyase (PAL), phosphoenolpyruvate carboxylase (PEPC) and glutamine synthetase (GS) in young expanding leaves of tea plants supplied with varied external N concentrations. Bars are standard deviations (n=3~4). Different letters above columns indicate significant difference at p<0.05.

Nutrient concentrations

Table 2 presents concentrations of elements in young shoots, recently matured leaves and roots. Total C concentration in recently matured leaves increased at N₄ whereas it was not influenced by N in other tissue. Total N concentration increased and C/N ratio decreased constantly with increasing external N supply. Concentrations of K, Ca, Mn in recently matured leaves and Mg and Mn in fibrous roots were significantly decreased by N application. There was also a trend towards decreasing leaf Mg and root K (p > 0.05) with increasing N application.

Table 2 Concentrations of element in young shoots, recently matured leaves and fibrous roots of tea plants as affected by a varied nitrogen supply in the nutrient solution.

Data are means \pm standard deviations (n = 4)

	N supply (mmol L ⁻¹)			
	0.30 (N ₁)	0.75 (N ₂)	1.50 (N ₃)	4.50 (N ₄)
<i>Young shoots</i>				
C (mg g ⁻¹)	462.4 \pm 20.2	470.8 \pm 3.7	475.4 \pm 7.8	467.8 \pm 9.7
N (mg g ⁻¹)	29.2 \pm 2.4a	34.9 \pm 1.3b	42.6 \pm 1.3c	55.3 \pm 1.1d
C/N	15.9 \pm 1.2d	13.5 \pm 0.6c	11.2 \pm 0.5b	8.5 \pm 0.1a
<i>Recently matured leaves</i>				
C (mg g ⁻¹)	459.8 \pm 7.8a	455.0 \pm 14.4a	467.6 \pm 17.2a	489.5 \pm 1.7b
N (mg g ⁻¹)	18.2 \pm 1.7a	23.6 \pm 0.8b	34.3 \pm 2.9c	45.2 \pm 2.2d
C/N	25.4 \pm 2.1d	19.3 \pm 0.2c	13.7 \pm 1.1b	10.9 \pm 0.5a
K (mg g ⁻¹)	15.4 \pm 0.6d	13.2 \pm 0.4c	11.8 \pm 0.9b	10.7 \pm 0.7a
Ca (mg g ⁻¹)	7.3 \pm 1.1c	6.2 \pm 1.4bc	4.7 \pm 0.8ab	4.2 \pm 0.8a
Mg (mg g ⁻¹)	3.5 \pm 0.6	3.4 \pm 0.5	2.9 \pm 0.4	2.7 \pm 0.3
Mn (mg kg ⁻¹)	194 \pm 59c	126 \pm 19b	97 \pm 6ab	71 \pm 12a
<i>Fibrous roots</i>				
C (mg g ⁻¹)	451.6 \pm 12.2	461.1 \pm 11.7	465.9 \pm 12.9	456.0 \pm 8.6
N (mg g ⁻¹)	13.1 \pm 1.3a	16.6 \pm 1.6b	23.1 \pm 1.6c	34.1 \pm 2.5d
C/N	34.6 \pm 2.6d	27.9 \pm 2.7c	20.3 \pm 0.9b	13.4 \pm 0.8a
K (mg g ⁻¹)	14.3 \pm 2.2	14.1 \pm 1.8	12.1 \pm 1.2	11.9 \pm 1.0
Ca (mg g ⁻¹)	2.1 \pm 0.3	2.3 \pm 0.2	2.4 \pm 0.4	2.3 \pm 0.1
Mg (mg g ⁻¹)	3.3 \pm 0.7b	3.3 \pm 0.4b	2.6 \pm 0.3a	2.0 \pm 0.1a
Mn (mg kg ⁻¹)	51 \pm 4b	46 \pm 9b	48 \pm 15b	31 \pm 7a

Different letters following data within a line indicate significant ($p < 0.05$) difference by LSD.

Free amino acids in plant tissue

With increasing N supply, fibrous roots accumulated significantly larger amounts of free amino acids (Figure 2A). Concentration of total free amino acid rose by 7-fold in N₄ treated roots compared to N₁. Similarly in young shoots, concentration of total free amino acid increased linearly with increasing external N supply (Figure 3A). The proportion of N in free amino acids to total tissue N concentration increased significantly with increasing external N supply, from 9.2% at N₁ to 15.2%, 21.9% and 29.8% for roots and from 2.4% at N₁, to 3.1%, 5.3% and 10.9% for young shoots at treatment levels N₂, N₃, and N₄, respectively.

The predominant free amino acid was Thea, which in roots accounted for 68-79% and in young shoots for 24-45% of total free amino acid (Figure 2B, 3B). Other free amino acids occurring abundantly in roots were arginine (Arg), glutamine (Gln) and glutamate (Glu), accounting for 3-11%, 1-7%, and 3-12% of the total (Figure 2C-2E). In young shoots Arg, Gln, and Glu accounted for 3-15%, 13-19% and 12-24% of total free amino acid concentration (Figure 3C-3E). However, the ratios of individual free amino acids to the total varied in three different patterns with external N supply. The first pattern was observed in Thea, of which proportion increased up to the N₃ treatment then decreased at N₄. The second pattern was in those of Gln and Arg, increasing only slightly from N₁ to N₃ treatment but sharply at N₄ (Figure 2B-2D; 3B-3D). The third pattern was found in Glu and other amino acids with consistently

decreasing proportional ratios with increasing external N supply (Figure 2E, 2F; 3E, 3F). Free phenylalanine (Phe) in young shoots increased significantly only in the N₄ (24%) treatment compared to N₁. The increase magnitude of Phe was much smaller than those of the predominant amino acids mentioned above.

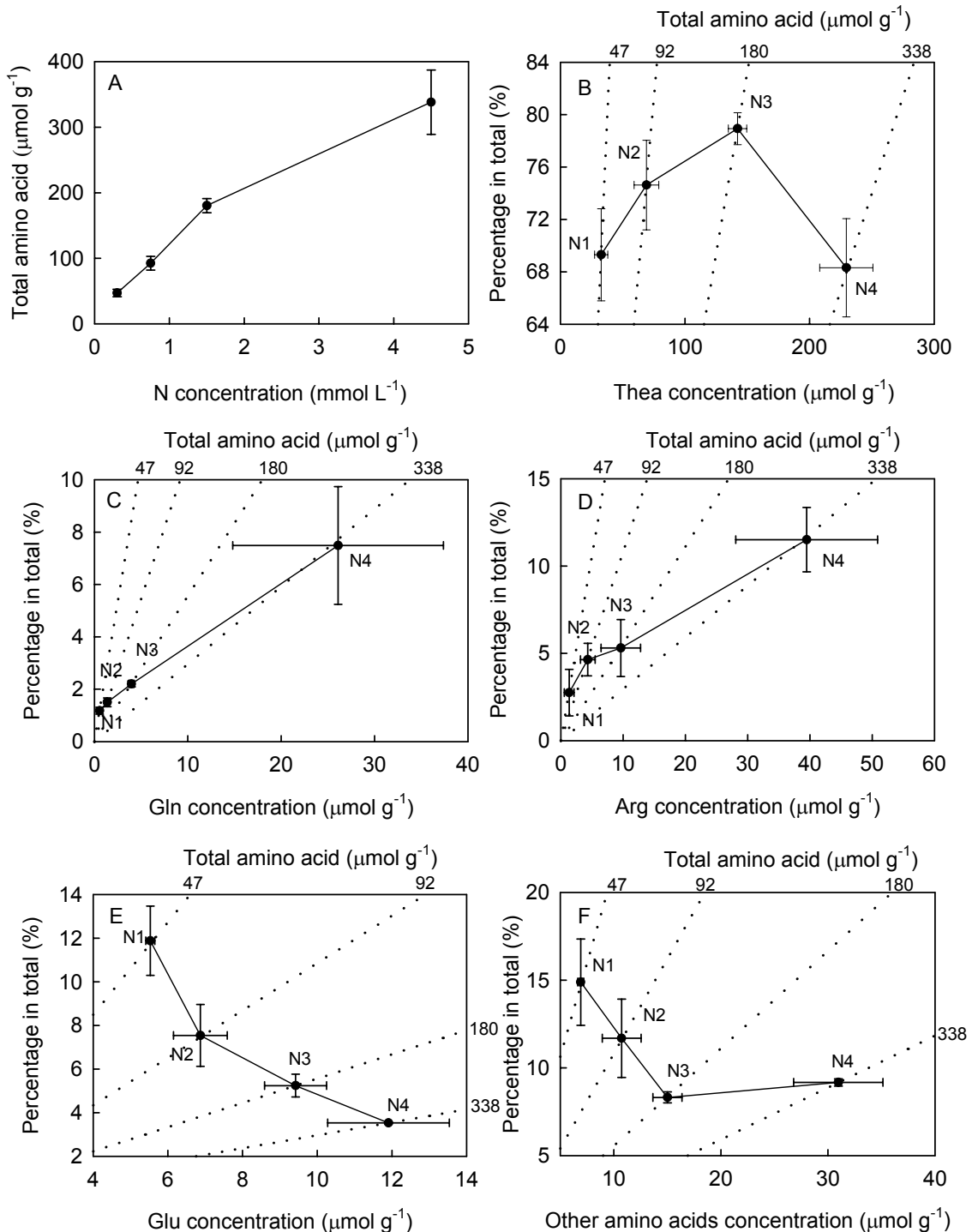


Figure 2 Concentrations of total (A), major free amino acids and their ratios to the total (B-F) in fibrous roots of tea plants supplied with varied external N concentrations.

Bars are standard deviations (n=4).

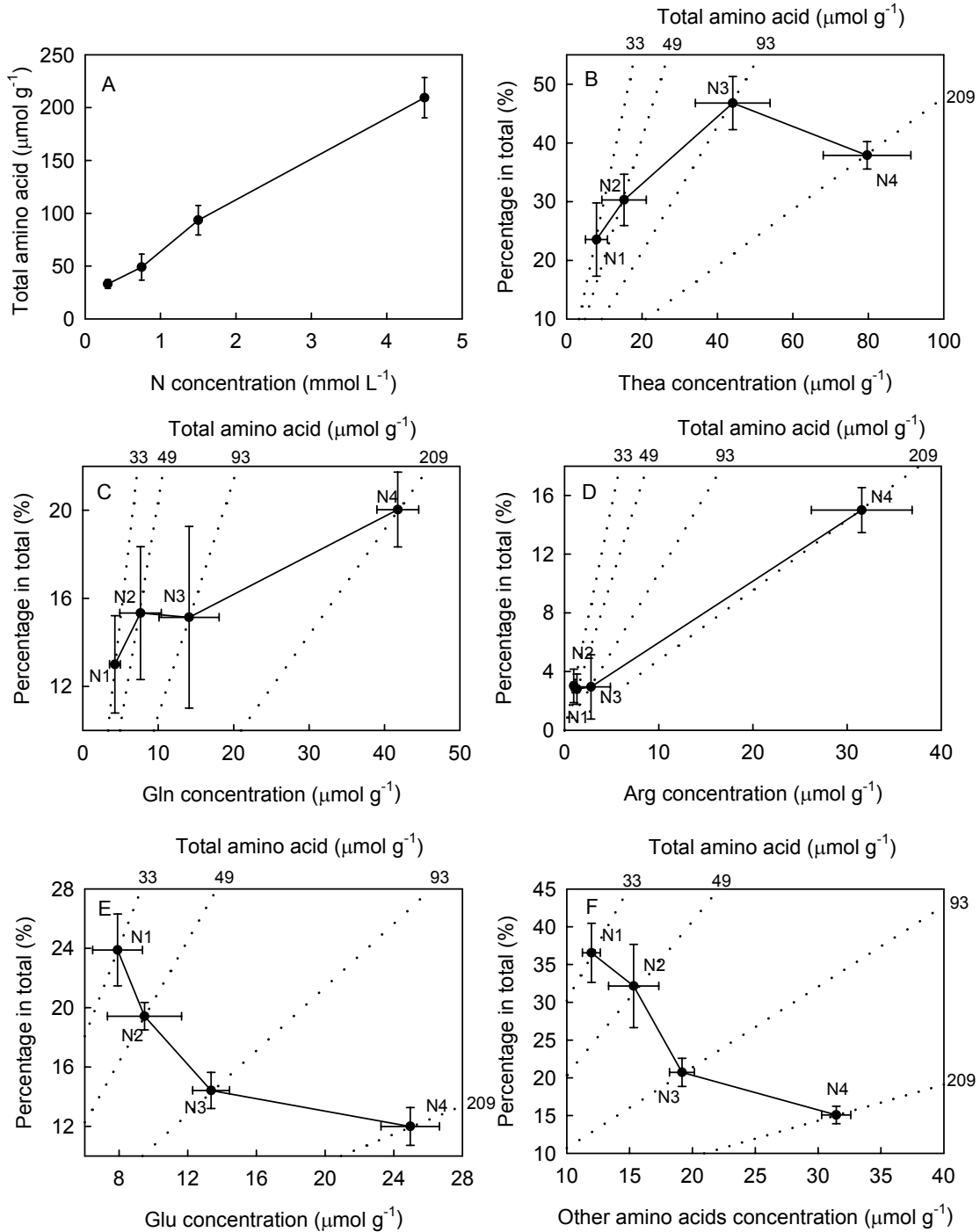


Figure 3 Concentrations of total (A), major free amino acids and their ratios to total (B-F) in young shoots of tea plants supplied with varied external N concentrations. Bars are standard deviations (n=4).

Sugars in plant tissues

Glucose and fructose concentrations in young shoots and recently matured leaves were significantly reduced with the N₄ treatment (Figure 4). Sucrose in young shoots was unaffected but in recently matured leaves it was larger in the two intermediate (N₂, N₃) than in the lowest or highest (N₁, N₄) N supplied levels. In roots, glucose concentration increased with increasing N supply whereas fructose was reduced at N₄ and sucrose decreased constantly as N supply increased.

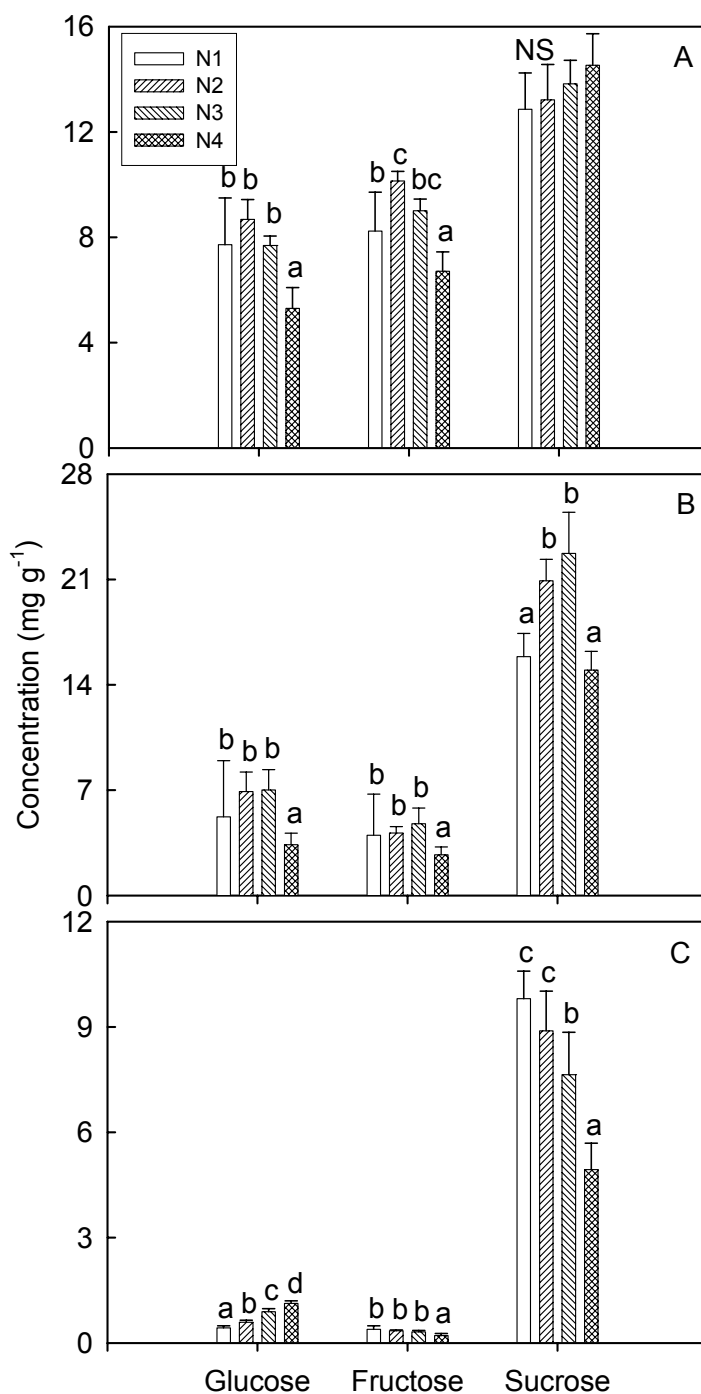


Figure 4 Concentrations of sugars in young shoots (A), recently matured leaves (B) and fibrous roots (C) of tea plants supplied with varied external N concentrations. Bars are standard deviations (n=4). Different letters above columns indicate significant difference at $p < 0.05$.

Secondary metabolites in young shoots

Only the highest N treatment significantly reduced concentration of total phenol in young shoots (Table 3). The major catechins were (-)-EGEC, (-)-EGC, (-)-ECG, and (-)-EC, accounting for 68-76% of the total phenol. Concentrations of (-)-EGCG and (-)-EGC increased slightly ($p > 0.05$) at the N₃ level. The N supply at the N₄ level reduced (-)-EGC, (-)-ECG and (-)-EC concentrations by 17%, 30% and 38%,

respectively, but not (-)-EGCG concentration as compared to N₁. Concentration of caffeine increased with increasing N supply.

Table 3 Total phenol, catechins and caffeine concentrations (mg g⁻¹) in young shoots of tea plants as affected by a varied nitrogen supply in the nutrient solution.

Data are means ± standard deviations (n = 4)

	N supply (mmol L ⁻¹)			
	0.30 (N ₁)	0.75 (N ₂)	1.50 (N ₃)	4.50 (N ₄)
Total phenol	199.4±6.7b	204.2±7.8b	194.9±8.6b	167.2±3.4a
(-)-EGCG	72.33±5.29	76.83±8.80	83.54±3.60	73.87±1.74
(-)-EGC	27.51±3.48b	27.51±2.45b	31.08±1.86b	22.84±1.17a
(-)-ECG	23.35±1.53b	22.90±1.65b	22.13±0.95b	16.25±0.58a
(-)-EC	13.11±0.76b	12.69±0.72b	12.23±0.94b	8.17±0.46a
(+)-C	1.81±0.23b	1.92±0.10b	1.82±0.16b	1.45±0.09a
Gallic acid	1.38±0.30	1.34±0.32	1.18±0.18	1.60±0.17
Total catechins	139.49±10.83b	143.20±12.35b	151.98±6.99b	124.18±2.46a
Caffeine	23.19±2.42a	29.02±0.89b	33.22±1.48c	37.52±1.12d

Different letters following data within a line indicate significant (p<0.05) difference by LSD.

Discussion

Plant growth, biomass allocation and nutrient uptake

Compared to field crops, demand for N fertilizers of tea plants, is relatively high. This is explained by the fact there are frequent harvests of tender young shoots containing high N content and in addition, regular pruning, removing large amounts of biomass (leaves, twigs), is carried out to maintain a vigorous vegetative growth. Therefore, N fertilizers are widely and extensively used in tea plantations. When yield response is observed, it has been attributed to an increase of shoot population density (number of shoots per unit area) and not to faster growth of young shoots (Owuor and Odhiambo, 1994). This observation is consistent with results of the present work, showing that only the number of young shoots per plant, but not weight per shoot, was affected by N levels, using identical sampling standards (one bud and two young leaves).

Root growth was unaffected whilst stems and leaves were enhanced by increasing N supply, leading to marked decrease in the contribution of root fraction to total plant weight. A relative reduction of root fraction under high N supply has been frequently observed (Wingler et al., 1994) and was explained as less carbon allocation to roots (Anandacoomaraswamy et al., 2002). In the present study total C content in roots was not influenced by N supply. However, root sucrose and fructose concentrations decreased with increasing N supply whereas glucose concentration increased. This possibly indicates that a large turnover from sucrose to glucose has occurred to meet the increasing demand for carbon skeletons and energy by assimilation of high N supply. The predominant N form supplied in nutrient solution in this experiment was NH₄⁺, due to clear preference for NH₄⁺ nutrition by tea plants (Chapter 1). It is thus anticipated that most absorbed N would be in this form, especially under large external N supply (e.g. N₄). Assimilation of NH₄⁺ occurs

in roots and requires substantial ketoglutarate, obtained from glucose (finally sucrose transported from the leaves) through the glycolytic and Krebs cycle pathways (Huppe and Turpin, 1994). Consequently the relative reduction of root fraction by high N supply could be due to large consumption of carbon for inorganic N (mainly NH_4^+) assimilation at the expense of growth.

Concentrations of leaf K, Mg, Ca and Mn diminished with increasing N supply whilst leaf K decrease was so pronounced that deficiency symptoms appeared. Decreased concentrations of these nutrients in leaves may be explained by a dilution effect or could be due to competitive inhibition by NH_4^+ (Marschner, 1995). Results clearly showed that nutritional imbalance was induced by high N supply.

Primary and secondary metabolites related with tea quality

Large N supply reduced concentrations of total phenol and catechins in young shoots, which agrees with previous findings in tea (Cloughley, 1983; Hilton et al., 1973; Owuor et al., 1991) and many other woody plants (e.g. Keinänen et al., 1999; Koricheva et al., 1998). In addition, the presented results showed concentrations of (-)-EGCG and (-)-EGC increased slightly ($p > 0.05$) in the moderate N treatment (N_3) whereas concentrations of total phenol and the major catechins (-)-EGC, (-)-ECG, (-)-EC were significantly reduced only by the largest N supply (N_4). This observation suggests that biosynthesis of catechins is possibly variable depending upon N status of plants and helps to clarify that earlier contradicting findings (see Introduction) might arise from the fact that experiments were carried out under completely different N supply levels.

Although decrease of phenolic compounds resulting from N fertilization has been demonstrated in a number of plant species, the mechanism behind this has not been clearly elucidated, being explained only by several hypotheses. Catechins are synthesized via phenylpropanoid pathway from Phe, which also serves as a substrate for synthesis of protein. One hypothesis thus, assumes that biosynthesis of phenolic compounds may compete with protein synthesis for Phe and may be inhibited because of limiting Phe availability under conditions of rapid incorporation into protein (da Cunha, 1987; Jones and Hartley, 1999; Margna, 1977). However, the present results show that in young shoots, concentration of free Phe increased with increasing N supply, although to a much smaller degree compared to those observed in other amino acids such as Thea, Gln, and Arg. On the other hand, the first step of the phenylpropanoid pathway is deamination of Phe to form cinnamic acid catalyzed by phenylalanine ammonia-lyase (PAL). Some experiments have shown a quantitative relationship between activity of PAL and accumulation of phenolic compounds either in tea or other plant species (Bate et al., 1994; Jeyaramraja et al., 2003). Thus, this enzyme is considered as a potential site for pathway regulation (Bate et al., 1994). It was suggested that N deficient plants increase the availability of ammonia by enhancing PAL activity, leading to accumulation of phenolic compounds (Margna, 1977). However, the present results showed that whilst PAL activity increased, concentrations of catechins were decreased by N application anyway. Sanchez et al. (2000a, b) also observed higher PAL activity in French bean plants in response to increasing N supply, covering a whole range from deficient

through optimal supply to toxic levels. These authors, however, found increasing accumulation of total phenol with increasing N supply. The inconsistent change of catechin concentrations with PAL activity reported here could possibly be explained by the fact that biosynthesis of catechins might be controlled by other enzymes in the pathway such as chalcone synthesis (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin reductase (LCR) (Park et al., 2004). Furthermore, carbon that directly forms catechins originates only partly from Phe with another part from malonyl-CoA during formation of chalcone. This indicates that accumulation of catechins may be directly affected by competition for carbon with other metabolisms.

Structurally flavonoids are carbon-based metabolites. It is assumed their production is determined by availability of carbohydrates (Bryant et al., 1983). Increasing N supply often leads to decrease of carbohydrate status in plants (Champigny et al., 1992). To verify whether production of catechins was limited by a low level of carbohydrates in plants under high external N supply, the concentrations of soluble reduced sugars were analyzed. It was observed that concentrations of major catechins (-)-EGC, (-)-ECG, (-)-EC in young shoots decreased significantly with a concomitant reduction of glucose and fructose concentrations with the N₄ treatment, suggesting production of these catechins was possibly linked to the availability of sugars, although concentration of sucrose (the major soluble sugar) remained unchanged.

The young shoot is a physiological 'sink' and has to import carbon (C) from a 'source' owing to its low photosynthetic capability. Translocation of carbohydrates from source tissue provides required resources for production of phenolic compounds (Arnold et al., 2004). Decrease of catechins synthesis in young shoots under ample N supply therefore, could be a result of low carbohydrate availability due to reduced photosynthate import from the 'source'. Recently matured leaves have committed photosynthetic competence thus, represent a 'source'. Photosynthate export out of the 'source' depends to a certain degree upon its strength. The resource strength of these leaves was then assessed by determining concentrations of soluble sugars. The two intermediate N treatments increased sucrose concentrations, possibly indicating improvement of photosynthetic capability. However, further increase of N supply (N₄) led to declining glucose and fructose concentrations, which could be a result of increasing export to sinks (young shoots and roots) in combination with an enhanced local consumption. An enhanced local consumption of sugars for N metabolism in recently matured leaves is evidently observed from their increasing total N content and a larger pool of free amino acids (data not shown) together with larger total C concentration. Furthermore, on a whole-plant level, roots could strongly compete for C with young shoots when C demand by roots was raised for increased NH₄⁺ assimilation under abundant N supply. Quantity and rate of C flow into young shoots was not measured, but it is likely import was not reduced given similar contents of total C in young shoots of the various N treatments. Another factor contributing to a low C availability for formation of catechins in young shoots is *in situ* competition from other, e.g. amino acid and protein metabolism. The enzyme assay showing greater activity of PEPC and GS under abundant N supply, provides strong evidence for such *in situ* C competition. As

mentioned above, the major part of N absorbed and assimilated by plants was in the form of NH_4^+ which occurs mainly in roots. Larger GS in young leaves of plants supplied with increasing N therefore was more likely related to intensified re-assimilation of NH_4^+ released from photorespiration (Lam et al., 1996) and catabolism of Thea particularly for tea plants. A part of Thea transported to young shoots hydrolyzes into glutamic acid and ethylamine (Tsushida, 1987). The latter is further oxidized to acetaldehyde by amine oxidase releasing NH_4^+ , which is possibly re-assimilated via the GS-GOGAT pathway in leaves. On the other hand, NH_2 -group of Gln translocated from roots can be transferred directly to other amino acids in young shoots. All these biochemical processes would impose strong demand for carbon skeletons in young shoots, further supported by the finding of greater activity of PEPC in plants with sufficient N supply. PEPC, a ubiquitous cytosolic enzyme, catalyzes carboxylation of PEP to form oxaloacetate. This enzyme provides anapleurotic carbon to replace TCA cycle intermediates consumed in NH_4^+ assimilation as well as amino acid biosynthesis and is a key enzyme connecting and regulating C allocation between primary C and N metabolisms (Huppe and Turpin, 1994; Champigny and Foyer, 1992). Increase of PEPC and GS activity in young leaves are important indicators of larger demand of C skeletons for synthesis of amino acids (Champigny and Foyer, 1992; Sugiharto and Sugiyama, 1992; Wingler et al., 1994). In addition, the C/N ratio in young shoots decreased significantly while total C remained unchanged with increasing N supply, suggesting that more C is partitioned to compounds containing N. Therefore, decrease of catechins biosynthesis in young shoots with increasing N supply probably arose from a diversion of carbon flux to N metabolism, reflected in substantially increased concentrations of free amino acids.

This study showed that accumulation of free amino acids in young shoots depended largely on N nutrition status, decreasing significantly in plants supplied with inadequate N. Other studies have also shown that under conditions of high N availability, free amino acids or proteins in plant tissue increase substantially (Näsholm and Ericsson, 1990; Warren and Adams, 2000). However, in the present experiment, biosynthesis and partitioning of N among amino acid fractions were differently affected by changing N supply. To illustrate these changes graphical vector analysis, by plotting absolute concentration of individual amino acid against its ratio to total free amino acid (sum of whole free amino acids), was carried out. The graphical vector analysis was originally developed for diagnosing nutrient limitations in trees (Timmer and Stone, 1978) and used in interpreting variation in plant allelochemistry (Koricheva, 1999). Obviously synthesis of Thea augmented greatly and those of Gln and Arg only slightly when external N supply rose from N_1 to N_3 as both their absolute concentrations and ratios increased. Further increasing N supply beyond N_3 still promoted synthesis of Gln and Arg, but not Thea. The ratio of Thea to the concentration of total free amino acid decreased as a result of a dilution effect as indicated by the fact that its absolute concentration was still increasing. Glu and other amino acids (excluding Thea, Gln, Glu and Arg) were constantly diluted by greater N supply (still showing increasing absolute concentrations). These results suggest that under excessive N supply, a relatively larger proportion of N was allocated to synthesis of Gln and particularly Arg than to Thea. In a previous

experiment high concentration of Arg in young shoots of tea plants was also detected under saturated N supply (Okano et al., 1997). Such an alteration pattern of free amino acid is possibly due to an imbalanced C : N status and may not be simply considered as an indicator of a disorder in the N metabolism as suggested by Okano et al. (1997). Excessive N availability over limiting carbon supply in roots (indicated by a low C : N ratio and significantly reduced sucrose and fructose contents) induced synthesis of amino acids with lower C : N ratios to improve the C economy. Arg has an apparent advantage as a low C : N ratio amino acid (6 : 4) over Thea with a high C : N ratio (7 : 2). It has been frequently shown that Arg accumulates to high concentrations in woody plants supplied with sufficient N (Näsholm and Ericsson, 1990; Warren and Adams, 2000). Nevertheless, accumulation of Arg in plants supplied with N₄ could also be due to a nutrient imbalance as indicated by K deficiency and a low tissue Mg status (see Table 2). It has been previously shown that application of K and Mg decreased Arg accumulation in the needles of heavily N-fertilized pines suffering from K and Mg deficiencies (Edfast et al., 1996).

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Chapter 4

Effect of counter anions (Cl^- vs. SO_4^{2-}) on concentrations of free amino acids in juvenile tea plants in pot experiments

Abstract

The effect of Cl^- as a counter anion compared to SO_4^{2-} on concentrations of free amino acids in young tea plants was investigated in pot experiments. In the first experiment, tea plants were grown in pots with soil and supplied with different levels of K as K_2SO_4 or KCl. Analysis of samples during a period of two years showed concentrations of free amino acid in young shoots increased only when K_2SO_4 was supplied. It was unchanged in the first year or decreased in the second when supplied with KCl at the same K level as in the K_2SO_4 treatment. The decrease of total free amino acid was mainly caused by a reduced concentration of theanine, likely due to Cl accumulation in plants. Concentrations of other nutrients in plant tissues were not influenced. The absorption and effect of Cl on nitrogen uptake were further investigated in a second experiment, in which soil-cultivated tea plants were supplied with varying amounts of Cl as KCl supplemented with NH_4Cl . Application of Cl induced foliage injury, and reduced the yield of young shoots. Severity of leaf damage was related to concentration of Cl in leaves. N uptake (measured with ^{15}N) of plants was reduced by Cl addition. NO_3^- accumulated in plant tissue was likely due to depressed reduction by Cl. To verify whether decrease of free amino acids was simply a result of inhibited NO_3^- assimilation, a third experiment where tea plants were cultivated exclusively in NH_4^+ nutrition with Cl (at the same concentration as NH_4^+) or without Cl was conducted. Similarly, concentrations of theanine and total free amino acid in young shoots were reduced by Cl. The result indicated that Cl likely has inhibitory effect on N metabolism of tea plants in addition to that on NO_3^- reduction. However, concentration of theanine in roots, where it is synthesized, was not influenced by Cl. Concentrations of total N in roots and mature leaves, the specific absorption rate of NH_4^+ and activity of glutamine synthetase in fibrous roots and young leaves were all unaffected by Cl as well. Based on the data, it is suggested that translocation of theanine from root to shoot and its further catabolism in young shoots might be influenced by Cl. The results are discussed in comparison with those from field experiments which did not show any significant Cl effect from KCl application compared to K_2SO_4 in terms of different Cl absorption and accumulation.

Introduction

Chloride is an essential micronutrient for higher plants with functions in photosynthesis, stomata operation and osmoregulation (Marschner, 1995). For most soils, inputs from various sources, such as rainwater, fertilizer application, irrigation water, dust and air pollution, supply sufficient Cl^- for plant growth and Cl deficiency rarely occurs (Xu et al., 2000). In contrast, excessive Cl supply and accumulation in plant tissues is a major obstacle restricting crop productivity in saline regions. In some sensitive crops Cl toxicity is induced by application of fertilizers containing Cl or irrigation of low-salinity water (Parker et al., 1983; Bar et al., 1997). Chloride accumulation to toxic concentrations in plant tissue causes anatomical disarrange-

ment of leaf cells leading to leaf damage and abscission, and reduces photosynthesis rate and finally plant growth (Bañuls and Primo-Millo, 1992; Gómez et al., 1998; Romeri-Aranda et al., 1998). The mechanism of Cl toxicity has been primarily attributed to 'ion excess' (Greenway and Munns, 1980), either in cell walls inducing osmotic stress (Oertli, 1968) or in the cytosol affecting a number of metabolic processes such as protein synthesis and enzyme activity (Gibson et al., 1984; Greenway and Munns, 1980). In addition, a high external Cl supply may cause nutritional imbalance by inducing osmotic stress decreasing transpiration or/and ionic competition (Marschner, 1995). For instance, it is frequently demonstrated that nitrate uptake and subsequent reduction are inhibited by Cl toxicity (Cerezo et al., 1997; 1999; Lea-Cox and Syvertsen, 1993; Romeri-Aranda et al., 1998; Ruiz and Romero, 2001).

Tea plants are widely cultivated in humid tropical and subtropical areas. Either deficiency or toxicity of Cl in tea has rarely been reported. However, Cl toxicity may occur under some specific conditions, for example under fertilization with fertilizers containing Cl. Application of NH_4Cl at high amounts induces leaf injury in the fields, attributed to Cl toxicity (Wu and Ru, 1975). Leaf damage occurs when Cl beyond 200 kg ha^{-1} is applied and becomes more severe with increasing application amount of NH_4Cl . Accumulation of free amino acid in young tea shoots was significantly reduced by application of KCl compared to K_2SO_4 in a pot experiment (Ruan et al., 1998). This has been interpreted as an indication of quality deterioration as free amino acids are the chief contributors to the mellowness taste of green tea (Chen et al., 1985; Mukai et al., 1992; Wang et al., 1988). The negative effect of Cl was explained as an antagonistic effect on NO_3^- uptake and subsequent assimilation (Ruan et al., 1998). Validation of this hypothesis, though being supported by the observation of decreasing activity of nitrate reductase in plants receiving Cl (Ruan et al., 1998), has not been vigorously investigated.

The work reported herein, consisting of three pot experiments, aimed at investigating Cl uptake and distribution in tea plants in response to varying external Cl supply and the effect of Cl on absorption of nutrients and accumulation of free amino acids.

Materials and Methods

Experiment 1

This experiment was conducted at Hangzhou, China to evaluate the effect of chloride on tea plant growth and concentrations of quality-related components. Each of four rooted cuttings *cv.* Longjing 43 was planted in pots filled with 5.5 kg air-dried soil in April 1999. The soil was placed on 2.5-cm thick quartz sand in the bottom. Each pot had a hole on the sidewall close to the bottom for drainage of excess rainwater. Three soils (designated as A, B, C hereafter) collected from different tea fields were selected because of their different K status. Contents of exchangeable K ($1 \text{ mol L}^{-1} \text{ NH}_4\text{OAC}$, pH 7.0) for soils A, B and C were 47, 92, and $184 \mu\text{g g}^{-1}$, respectively. The soils had pH values of 4.29, 3.91, 4.19 and contained organic matter (mg g^{-1}) 7.5, 11.5, and 9.7, respectively. Three levels of K (K_0 , $\text{K}_1\text{-S}$, $\text{K}_2\text{-S}$, corresponding to a total of 0, 2.24 and 4.48 g K/pot for experimental period,

respectively, were applied as K_2SO_4 and KCl at K_2 level (K_2 -Cl) together with identical amounts ($g\ pot^{-1}$) of N (5.19) and P (1.13) as $(NH_4)_2SO_4$ and $NH_4H_2PO_4$. The total amount of Cl applied in the experimental period was $4.08\ g\ pot^{-1}$ for the KCl treatment. S ranged from 5.34 to $7.18\ g\ pot^{-1}$ depending on the K levels and source. Nutrients prepared in solution were divided into applications at one-week interval from April to September in 2001 and 2002. There were four replications for each treatment. Plants were placed in open air and watered with tap water when necessary. The young shoots consisting of one bud and two leaves were harvested in years 2001 and 2002, steamed for 2 min and oven-dried at $70^\circ C$. In July 2002, mature leaves developed from spring growth of the same year (hereafter referred to as mature spring leaves) were identified according to their positions on the branches and randomly collected. These leaves were oven-dried and ground for further analysis.

Concentration of total free amino acid in young shoots was determined with routine methods after extracted by distilled H_2O in approximate $90^\circ C$ water bath for 45 min with occasional shaking by hand (Tea Research Institute, 1983). The extract was immediately filtered and cooled in tap water. Concentration of total free amino acid was analyzed by ninhydrin reaction in the presence of $SnCl_2$ at pH 8.0. Composition of free amino acids was measured by HPLC with AccQ Tag column ($3.9\ mm \times 150\ mm$) and a fluorescence detector after derivatization with AccQ-Fluor Reagent Kit following the manufacturer's protocol (Waters Corporation, 1996).

NO_3^- , Cl^- , SO_4^{2-} and organic anions in plant samples were extracted with H_2O for 10 min in $100^\circ C$ water bath and their concentrations measured by ion chromatography (DX 300, Dionex, Idstein, Germany).

Experiment 2

This pot experiment was conducted to investigate Cl absorption and effect on N uptake by tea plants in soil supplied with varying Cl amounts. Each pot contained 4 kg medium (mixture of soil and perlite). Planting was with 1 rooted-cutting *cv.* Longjing 43. Plants were cultivated under low N availability ($1.05\ g\ N\ pot^{-1}$) for two years prior to the present experiment. There were four treatments supplied with different amounts of Cl at 0, 0.76, 1.14, and $1.52\ g\ pot^{-1}$ (corresponding to 0, 190, 285 and $380\ mg\ kg^{-1}$ soil, hereafter referred to as Cl_0 , Cl_1 , Cl_2 and Cl_3). Chloride was supplied as KCl and supplemented with NH_4Cl in Cl_2 and Cl_3 treatments. All pots received identical amounts ($g\ pot^{-1}$) of N (1.0), P (0.44), K (0.83), Mg (0.15) but variable S (0.89 in Cl_0 and 0.55 in others). There were 4 replications for treatments (except three replications for Cl_2 treatment because of loss of one pot). The nutrients were divided into 2 applications with the first in mid-March and the second 10 days later. For each application, nutrients were dissolved in 200 mL deionized water and poured to the soil surface followed by additional 100 mL water. In the second application, ^{15}N labeled $(NH_4)_2SO_4$ (^{15}N abundance=10%) was used. The experiment was conducted at Kiel, Germany in a glasshouse supplied with additional light (SON-T AGRO 400 W, Philips) to ensure minimum light intensity of $220\ \mu mol\ m^{-2}\ s^{-1}$ at canopy level. Watering was with deionized water. The maximum air temperature in the glasshouse was approximately $34\ ^\circ C$ (daytime) and minimum $20\ ^\circ C$ (night). Relative humidity was maintained around 70% by a humidifier. Young shoots

consisting of one bud with two unfolding leaves were collected 4 times with the first sampling at 5 days after the second nutrient application. The whole plant was sampled 15 days after the second nutrient application, separated into mature leaves and stems. A portion of fibrous roots was recovered by washing the soil from the root system. Plant samples were oven dried and milled for further analyses. Concentrations of Cl and other anions in plant samples were analyzed as in Experiment 1. Total N and ^{15}N abundance were determined in an elemental analyzer (Carlo Erba, Milano, Italy) coupled to a mass spectrometer (Finigan Corp., Bremen, Germany). N_{dff} (N derived from the fertilizer) and the amount of ^{15}N absorbed by the plants were calculated according to the following equations:

$$N_{\text{dff}} (\%) = \frac{(^{15}\text{N} \text{ abundance in sample} - \text{the natural } ^{15}\text{N} \text{ abundance})}{(^{15}\text{N} \text{ abundance of the fertilizer} - \text{the natural } ^{15}\text{N} \text{ abundance})} * 100\%$$

$$^{15}\text{N} \text{ content in plant (mg pot}^{-1}\text{)} = N_{\text{dff}} * \text{total N content (mg g}^{-1}\text{)} * \text{biomass (g pot}^{-1}\text{)}$$

Experiment 3

This experiment was carried out to examine the effect of Cl on concentrations of free amino acids under supply of NH_4^+ in the nutrient solution. Three seedlings with 3-4 leaves germinated from seeds (*cv.* Longjing 43) were transplanted to 0.2 mmol L⁻¹ CaSO₄ solution for five days and to nutrient solution thereafter. The strength of the nutrient solution was increased stepwise from 1/3 to 2/5 (week 2 and 3), 1/2 (week 4), 3/4 (week 5 to 12) and finally to full strength. It contained macronutrients (mmol L⁻¹) N 1.7, P 0.07, K 0.67, Ca 0.53, Mg 0.67 and micronutrients ($\mu\text{mol l}^{-1}$) Zn 0.67, Cu 0.13, Mn 1.0, B 7, Mo 0.33, EDTA-Fe 4.2 and Al 70. In the control treatment (CK), a low Cl concentration (full strength 0.033 mmol L⁻¹) was supplied as CaCl₂ only to meet the physiological demand for Cl whereas in treatment with Cl (+Cl), its concentration at full strength was 1.7 mmol L⁻¹ supplied as KCl and MgCl₂. This Cl concentration, at similar range as N, was selected to avoid the induction of, if any, osmotic stress for the plants. Nitrogen was supplied as NH_4^+ . 3,4-dimethyl pyrazole phosphate (DMPP) at 1% of the N amount was included to inhibit any potential nitrification in the nutrient solution. The pH of the nutrient solution was maintained at 4.0 with H₂SO₄ and NaOH by means of pH stat system. Treatments were replicated 4 times. Each pot contained 4 L nutrient solution with three seedlings initially, thinned to two plants 12 weeks later. The nutrient solution was replaced every week. The experiment was carried out at Kiel, Germany for 20 weeks from May to September. Plants were provided with additional light (SON-T AGRO 400 W, Philips) to ensure a minimum light intensity of 220 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level from the beginning until 4 weeks after transplanting, thereafter, they received only natural light. Other conditions of the glasshouse were the same as described in Experiment 2. Young shoots consisting of one bud with two unfolding leaves were collected regularly, quickly frozen by liquid N₂ and freeze dried.

Specific absorption rate of NH_4^+ was determined by measuring its depletion in the nutrient solution four times, two over one-week intervals at week 10 and 11 and another two over 5-day intervals at week 18 and 19, respectively. NH_4^+ concentration was determined by indophenol blue method (Mulvaney, 1996). Absorption rate was expressed on a per root dry weight basis calculated from data at the final sampling

(week 20) and at the beginning (week 1) assuming a constant growth rate per week. Average rates from the four measurements are reported herein.

Chlorophyll content of mature leaves was measured by a portable chlorophyll meter (Minolta SPAD-502, Osaka) just before the final sampling (week 20). For each pot 10-12 leaves from the upper canopy were randomly selected. The averaged readings were taken for that pot (replicate). Chlorophyll meter readings were calibrated ($r^2 = 0.912$, $p < 0.0001$) with chlorophyll contents measured by the method of Arnon (1949) from a parallel experiment under similar growth conditions.

Glutamine synthetase (GS, EC 6.3.1.2) in fibrous roots and young expanding leaves (third leaf from the bud) was extracted with a buffer (pH 7.5) containing 50 mmol L⁻¹ Tris, 5 mmol L⁻¹ EDTA, 5 mmol L⁻¹ dithioerythritol at a ratio of 10 ml g⁻¹ FW and 5% (w/v) PVPP using a Potter S homogenizer cooled with ice (Gerendás et al., 1998). Extracts were centrifuged at 12,000 g for 10 min. GS activity was determined by synthetase assay (Magalhaes and Huber, 1991) and a 30-min incubation time for the reaction was adopted. One unit of enzyme activity corresponded to formation of 1 μ mol γ -glutamyl hydroxamate per gram fresh material per min.

The profile of free amino acids was analyzed as o-phthalaldehyde derivatives on a reverse phase C₁₈ column (Hypersil ODS, 3 μ m, 250 mm x 4.6 mm) using an automated HPLC system (Gerendás et al., 1998) after extraction with H₂O (1/50, w/v) in a 100°C water bath for 5 min. NO₃⁻, Cl⁻, SO₄²⁻ and organic anions in plant samples were extracted with chloroform : methanol (3:7, v/v) as previously described (Lohaus et al., 2000) and measured by ion chromatography (DX 300, Dionex, Idstein, Germany). For determinations of K, P, Mg, Ca, Mn, Fe, Cu, and Al, plant samples were ashed at 500°C and measured by Induced Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Model IRIS-AP, Thermo Jarrel Ash Corp., USA). Total N was measured on an element analyzer.

Statistical analysis

Data from experiments 1 and 2 were subjected to one-way analysis of variance and LSD test performed when initial F test was significant. For experiment 3, t-test was executed to examine the effect of Cl.

Results

Experiment 1

Yield of young shoots in 2001 was unaffected (data not shown) whereas it increased significantly at K₁ in 2002 on soils B and C, and on soil A (but $p > 0.05$) (Figure 1). A further significant yield response was observed at K₂ as K₂SO₄ in the same soils. Plants receiving KCl compared to K₂SO₄ produced similar yields of young shoots in soils A and C but significantly decreased yield on soil B.

Data in Table 1 present concentrations of anions in mature spring leaves. Concentration of Cl was increased by KCl application. Concentration of SO₄²⁻ increased with S (and K) application level and was larger in plants supplied with KCl than in the control (K₀) on two soils (B and C). NO₃⁻ concentration was extremely low

and was not influenced by K and/or Cl application. Citrate and malate concentrations were generally below 100 mg kg⁻¹. Oxalate was the predominant organic anion but its concentration changed inconsistently among soils, increasing with K application with either K source in soil B or only with KCl in soil C but was unaffected in soil A. Concentration of K increased with K application amount and correlated linearly ($r=0.70$, $p<0.001$) with the yield of young shoots in 2002. Concentrations of PO₄³⁻ and other elements (N, P, K, Mg, Ca, Mn, Fe, Cu and Al, data not shown) were not different between plants receiving the two K sources.

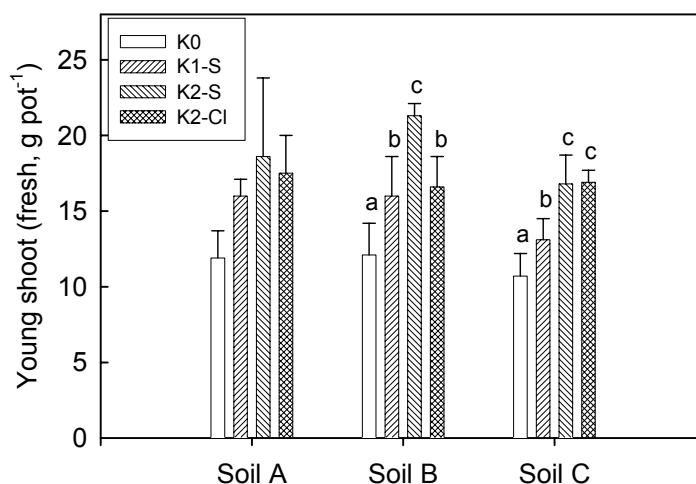


Figure 1. Production of young shoots of tea plants supplied with different potassium levels and sources (Experiment 1). Bars are standard deviations (n=4). Different letters above columns within the same soils indicate significant difference at $p<0.05$ by LSD test.

Table 1. Concentrations of anions in mature spring leaves of tea plants supplied with different potassium levels and sources (Experiment 1). Data are means \pm standard deviations (n=4).

Soil	Treatment	Cl ⁻ (mg g ⁻¹)	SO ₄ ²⁻ (mg g ⁻¹)	PO ₄ ³⁻ (mg g ⁻¹)	NO ₃ ⁻ (mg kg ⁻¹)	Oxalate (mg g ⁻¹)
A	K ₀	1.24 \pm 0.30a	1.30 \pm 0.32a	0.15 \pm 0.03	6 \pm 6	3.60 \pm 0.62a
	K ₁ -S	1.24 \pm 0.32a	2.32 \pm 0.53b	0.16 \pm 0.04	28 \pm 32	3.59 \pm 0.45a
	K ₂ -S	1.19 \pm 0.09a	3.47 \pm 0.65c	0.17 \pm 0.03	11 \pm 23	3.99 \pm 0.38a
	K ₂ -Cl	4.04 \pm 0.33b	2.85 \pm 0.27bc	0.20 \pm 0.06	1 \pm 1	5.01 \pm 0.34b
B	K ₀	1.45 \pm 0.08b	0.85 \pm 0.15a	0.60 \pm 0.16	18 \pm 22	1.36 \pm 0.52a
	K ₁ -S	1.21 \pm 0.06a	1.45 \pm 1.02ab	0.38 \pm 0.19	29 \pm 12	3.63 \pm 0.65b
	K ₂ -S	1.06 \pm 0.07a	3.29 \pm 0.38c	0.39 \pm 0.12	24 \pm 6	4.17 \pm 0.44b
	K ₂ -Cl	3.66 \pm 0.22c	2.33 \pm 0.47b	0.51 \pm 0.07	8 \pm 6	3.83 \pm 0.39b
C	K ₀	1.33 \pm 0.37a	1.27 \pm 0.55a	0.17 \pm 0.05	10 \pm 14	3.22 \pm 0.74
	K ₁ -S	1.06 \pm 0.17a	2.08 \pm 0.60ab	0.22 \pm 0.05	3 \pm 5	3.38 \pm 0.24
	K ₂ -S	1.73 \pm 1.31a	2.84 \pm 0.20b	0.17 \pm 0.10	13 \pm 26	3.72 \pm 0.68
	K ₂ -Cl	3.88 \pm 0.31b	1.61 \pm 0.85a	0.22 \pm 0.04	30 \pm 26	3.86 \pm 0.22

Different letters following data within columns of each soil indicate significant difference at $p<0.05$.

The effect of K and Cl application on concentration of total free amino acid varied within the soils in the two years, being statistically significant for soils A and

C in 2001, and soils A and B in 2002 (Figure 2). Compared to K_2SO_4 , the negative effect of KCl on concentration of total free amino acid was observed in both years. In 2002 the concentration of total free amino acid in plants receiving KCl was even lower than the control (K_0). The profile of free amino acids in young shoots was analyzed for summer tea of 2001 from soils A and C to determine which amino acids were affected. Theanine was the predominant amino acid and was the one mostly affected by KCl application (Table 2). Both its absolute concentration and ratio (percentage) to the total (sum of HPLC analyzed amino acids) were substantially decreased by KCl compared to K_2SO_4 . Other free amino acids, except Asp for soil A, were not influenced by KCl application.

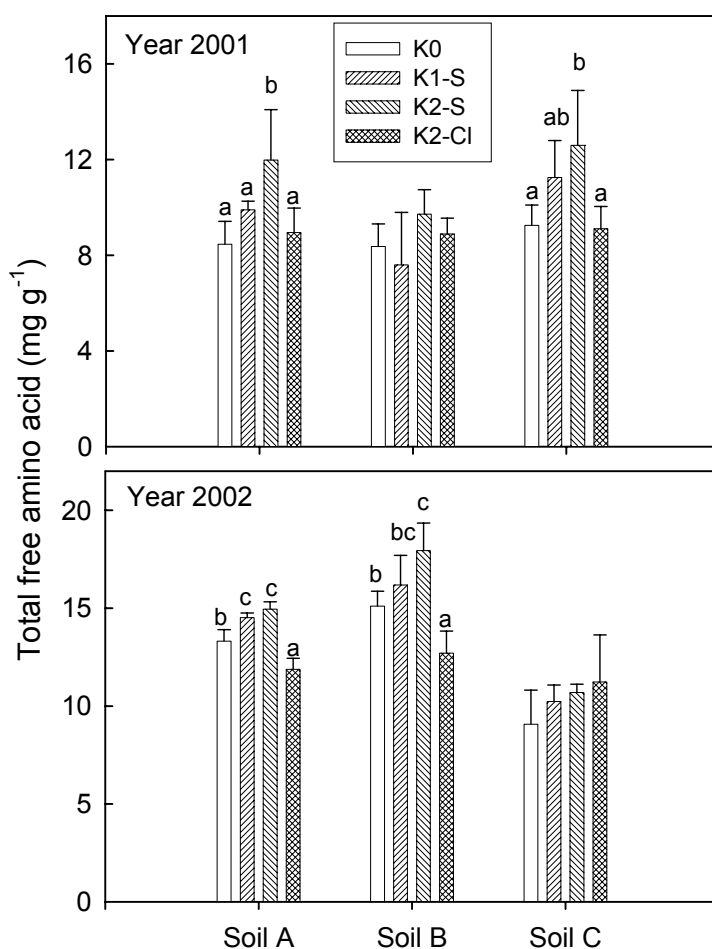


Figure 2. Total free amino acid in young shoots of tea plants supplied with different potassium levels and sources in year 2001 and 2002 (Experiment 1). Bars are standard deviations (n=4). Different letters above columns within the same soils indicate significant difference at $p < 0.05$ by LSD test.

Concentrations of polyphenols and caffeine in young shoots from the year 2001 were not influenced by K application (data not shown). In 2002, K application increased concentrations of polyphenols in plants grown on soil B ($p < 0.05$) and only slightly ($p > 0.05$) in plants from the other two soils. Caffeine in young shoots of 2002 significantly increased with K application in soils A and B (data not shown). The two K sources had similar effect on concentrations of polyphenols and caffeine.

Table 2. Concentrations of free amino acids ($\mu\text{mol g}^{-1}$) in summer tea in 2001 (Experiment 1).
Data are means \pm standard deviations (n=4).

Amino acid	Soil A				Soil C			
	K ₀	K ₁ -S	K ₂ -S	K ₂ -Cl	K ₀	K ₁ -S	K ₂ -S	K ₂ -Cl
Thea	27.37 \pm 5.08a	29.19 \pm 3.6a	39.55 \pm 5.25b	22.82 \pm 2.95a	25.88 \pm 4.20a	28.80 \pm 3.41a	36.15 \pm 5.51b	24.30 \pm 2.47a
Glu	4.88 \pm 3.52	4.58 \pm 0.59	5.12 \pm 0.51	4.80 \pm 1.76	7.30 \pm 2.21	9.44 \pm 1.22	7.44 \pm 0.44	7.69 \pm 2.38
Asp	3.26 \pm 1.13a	3.88 \pm 0.84a	5.65 \pm 1.40b	3.13 \pm 0.52a	2.99 \pm 0.97	4.60 \pm 1.01	3.39 \pm 0.79	5.17 \pm 2.11
Arg	0.88 \pm 0.66	0.38 \pm 0.09	0.84 \pm 0.25	0.79 \pm 0.26	0.83 \pm 0.53	0.90 \pm 0.33	0.71 \pm 0.29	0.63 \pm 0.16
Ala	1.08 \pm 0.24	0.85 \pm 0.16	1.25 \pm 0.20	1.13 \pm 0.33	1.75 \pm 0.55	1.67 \pm 0.19	1.47 \pm 0.20	1.81 \pm 0.11
Cys	3.25 \pm 0.22	3.37 \pm 0.31	3.36 \pm 0.18	3.21 \pm 0.15	2.83 \pm 0.20	3.04 \pm 0.36	2.69 \pm 0.24	2.94 \pm 0.42
Gly	0.20 \pm 0.25	0.21 \pm 0.19	0.26 \pm 0.18	0.15 \pm 0.23	0.33 \pm 0.40	0.31 \pm 0.35	0.23 \pm 0.39	0.54 \pm 0.14
His	1.10 \pm 0.35	0.76 \pm 0.22	1.77 \pm 0.55	1.30 \pm 0.68	1.79 \pm 0.62	1.50 \pm 0.31	1.74 \pm 0.41	1.87 \pm 0.64
Ile	0.44 \pm 0.20	0.44 \pm 0.03	0.46 \pm 0.05	0.51 \pm 0.09	0.82 \pm 0.13	0.77 \pm 0.03	0.70 \pm 0.04	0.69 \pm 0.06
Leu	0.42 \pm 0.24	0.35 \pm 0.04	0.39 \pm 0.06	0.47 \pm 0.14	1.09 \pm 0.12	0.92 \pm 0.15	1.00 \pm 0.02	1.02 \pm 0.06
Lys	0.57 \pm 0.18	0.75 \pm 0.16	0.64 \pm 0.20	0.60 \pm 0.09	0.88 \pm 0.05	0.86 \pm 0.10	0.59 \pm 0.22	0.88 \pm 0.09
Met	0.01 \pm 0.02	0.05 \pm 0.05	n.d.	0.15 \pm 0.29	n.d.	n.d.	n.d.	n.d.
Phe	0.36 \pm 0.11	0.37 \pm 0.04	0.49 \pm 0.10	0.48 \pm 0.10	0.87 \pm 0.08	0.78 \pm 0.07	0.89 \pm 0.05	0.71 \pm 0.06
Pro	0.40 \pm 0.24	0.33 \pm 0.06	0.44 \pm 0.13	0.45 \pm 0.11	1.63 \pm 1.26	1.87 \pm 1.48	1.11 \pm 0.67	0.72 \pm 0.05
Ser	7.71 \pm 6.93	5.23 \pm 1.87	6.92 \pm 0.47	6.06 \pm 1.82	3.61 \pm 0.76	3.41 \pm 0.41	4.69 \pm 1.21	3.78 \pm 0.33
Thr	1.04 \pm 0.48	0.67 \pm 0.08	0.84 \pm 0.17	1.04 \pm 0.38	4.59 \pm 2.69	2.98 \pm 1.72	5.65 \pm 0.70	6.92 \pm 2.93
Tyr	0.56 \pm 0.17	0.43 \pm 0.13	0.56 \pm 0.11	0.64 \pm 0.14	0.78 \pm 0.07	1.01 \pm 0.30	0.82 \pm 0.13	0.67 \pm 0.12
Val	1.31 \pm 0.46	1.19 \pm 0.32	1.76 \pm 0.44	1.50 \pm 0.17	2.95 \pm 0.73	2.68 \pm 0.83	2.99 \pm 1.75	2.80 \pm 0.80

n.d.: not detected. Different letters following data in the same line of each soil indicate significant difference at $p < 0.05$.

Experiment 2

In this experiment, Cl was applied during a relatively short time and induced Cl toxicity in plants. The injured plants showed scorched margins and yellowing spots in leaves of the upper canopy (Figure 3). Leaf damage was more severe in treatments receiving moderate (Cl₂) and high Cl (Cl₃) levels. In these two treatments some fully necrotic leaflets were observed. Cl toxicity was less severe and visible symptoms developed only on some leaves in the Cl₁ treatment due to the short time after treatment application. Foliage injury was related to Cl concentration in the leaves, which increased with Cl amounts added (Figure 4). High concentration of Cl accumulated mainly in leaves and young shoots whereas root Cl concentration responded insensitively to Cl application and represented the major portion of Cl in the plants without external Cl addition.



Figure 3. Pictures showing leaf injury following varying Cl application rates (Cl₁:190, Cl₂: 285 and Cl₃: 380 mg kg⁻¹ soil) (Experiment 2).

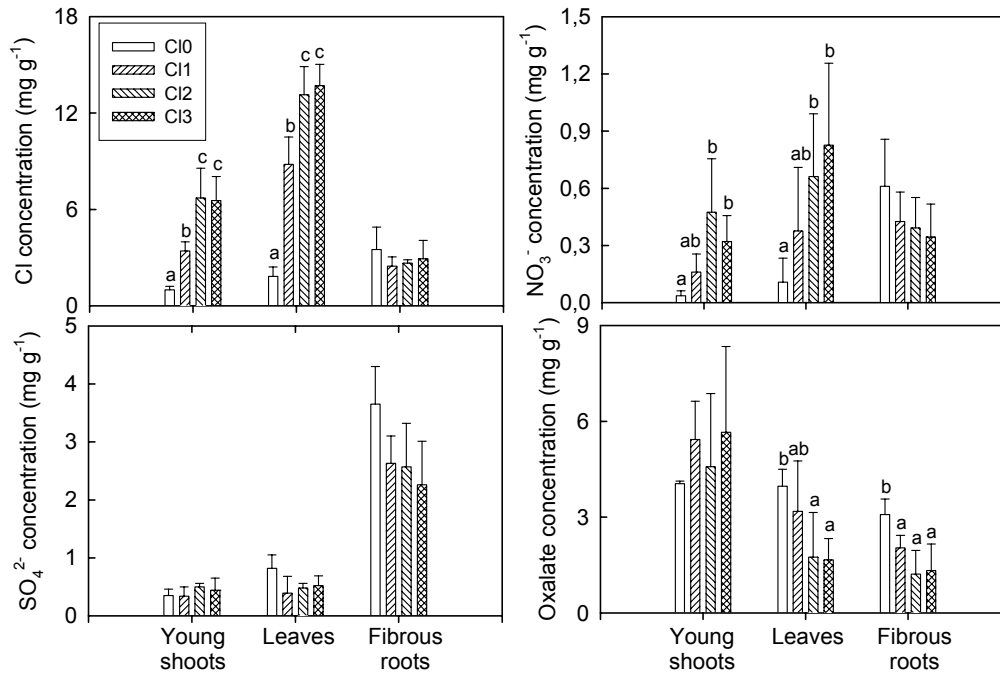


Figure 4. Concentrations of anions in plant tissues in response to varying Cl application rates (Experiment 2). Bars are standard deviations (n=3~4). Different letters above the columns indicate significant difference at p<0.05 by LSD test.

Table 3. Total N, N_{dff} and ¹⁵N content in plants supplied with varying Cl (Experiment 2). Data are means ± standard deviations (n=3~4).

	Organ	Treatment			
		Cl ₀	Cl ₁	Cl ₂	Cl ₃
Total N (mg g ⁻¹)	Young shoot	58.3±1.4	62.8±6.7	63.1±4.6	63.7±2.0
	Mature leaves	45.2±3.2	46.9±1.3	46.1±5.6	44.5±7.7
	Stem	13.8±0.4b	14.0±0.3b	12.2±0.8a	12.5±0.9a
	Fibrous root	18.1±1.8	16.7±2.8	15.2±0.9	13.9±1.9
N _{dff} (%)	Young shoot	9.04±2.10	8.40±1.35	6.69±1.51	6.57±0.83
	Mature leaves	5.09±1.06c	4.11±1.00bc	2.77±0.65ab	2.69±0.34a
	Stem	5.69±0.86b	3.94±1.00a	3.04±0.74a	2.92±0.46a
	Fibrous root	6.18±2.30b	4.36±1.07ab	3.65±1.43a	2.76±0.68a
¹⁵ N content (mg plant ⁻¹)	Young shoot (1)	8.1±2.9b	4.5±2.2a	2.4±0.3a	3.5±1.7a
	Mature leaves (2)	22.0±8.7b	14.4±4.4ab	8.6±2.7a	9.2±3.1a
	Stem (3)	6.8±2.1b	4.5±1.7a	2.8±0.4a	2.9±0.8a
	Sum (1+2+3)	36.9±13.1b	23.4±7.3a	13.8±3.3a	15.6±4.7a

Different letters following data in the same line indicate significant difference at p<0.05.

Yield of young shoots decreased significantly in all plants receiving Cl application whereas biomass of the above ground (stem + leaves) portion was unaffected (data not shown). Application of Cl reduced concentrations of oxalate in mature leaves and fibrous roots but did not significantly influence those of SO₄²⁻ (Figure 4), PO₄³⁻, malate or citrate (data not shown). Plants receiving the moderate and high Cl amounts accumulated significantly larger NO₃⁻ concentrations in young shoots and leaves. Total N contents were not affected by Cl (Table 3). N_{dff} and the amount of ¹⁵N in plants decreased significantly in Cl-treated plants. The distribution

profile of ^{15}N in plant organs (17.4-22.4% in young shoots, 59-62.3% in mature leaves and 18.4-20.3% in stems) was not influenced.

Experiment 3

Plant growth was not influenced by supplying Cl in the nutrient solution, although those receiving Cl tended to produce slightly ($p>0.05$) smaller roots and whole plant biomass production (Table 4). Chlorophyll content in mature leaves however, was significantly reduced by Cl. The specific absorption rate of NH_4^+ , activity of GS in fibrous roots and young leaves were not different between the treatments with and without Cl. Cl treatment increased Cl concentration in mature leaves but not in fibrous roots. Other nutrients and anions in mature leaves and fibrous roots were only marginally influenced (Table 5).

Table 4. Parameters of growth and NH_4^+ absorption in plants supplied with Cl (+Cl) and without (CK) (Experiment 3). Data are means \pm standard deviations (n=4).

Parameter		CK	+Cl
Dry matter (g pot ⁻¹)	Young shoots	1.55 \pm 0.49	1.89 \pm 0.38
	Root	3.79 \pm 0.82	2.52 \pm 0.70
	Whole plant	15.58 \pm 2.95	12.04 \pm 0.90
Chlorophyll ($\mu\text{g cm}^{-2}$)		252.6 \pm 8.4	228.7 \pm 11.1*
GS activity ($\mu\text{mol g}^{-1} \text{fw min}^{-1}$)	Leaf	1.34 \pm 0.06	1.54 \pm 0.29
	Fibrous root	0.35 \pm 0.03	0.42 \pm 0.07
NH_4^+ uptake ($\mu\text{mol g}^{-1} \text{root dw d}^{-1}$)		165.9 \pm 21.8	171.9 \pm 8.5

* indicates significant difference from CK at $p<0.05$ with t-test.

Table 5. Concentrations of elements and anions in plants supply with (+Cl) and without Cl (CK) (Experiment 3). Data are means \pm standard deviations (n=4).

Element/ Anion	Mature leaves		Fibrous roots	
	CK	+Cl	CK	+Cl
N (mg g ⁻¹)	46.6 \pm 5.7	50.2 \pm 2.4	39.7 \pm 7.3	42.0 \pm 5.7
P (mg g ⁻¹)	5.3 \pm 1.1	5.0 \pm 0.8	11.3 \pm 2.1	12.4 \pm 1.0
K (mg g ⁻¹)	7.5 \pm 1.2	8.0 \pm 1.2	12.9 \pm 1.3	12.2 \pm 0.7
Ca (mg g ⁻¹)	4.1 \pm 0.3	4.0 \pm 0.3	2.2 \pm 0.4	2.4 \pm 0.4
Mg (mg g ⁻¹)	2.5 \pm 0.3	2.6 \pm 0.2	2.1 \pm 0.4	2.2 \pm 0.5
Fe (mg kg ⁻¹)	282 \pm 183	310 \pm 406	257 \pm 165	145 \pm 51
Mn (mg kg ⁻¹)	113 \pm 14	125 \pm 10	40 \pm 29	64 \pm 23
Cu (mg kg ⁻¹)	13 \pm 8	7 \pm 4	46 \pm 11	38 \pm 9
Zn (mg kg ⁻¹)	40 \pm 13	33 \pm 7	410 \pm 182	536 \pm 57
B (mg kg ⁻¹)	57 \pm 11	62 \pm 13	8.0 \pm 0.3	6 \pm 1
Al (mg kg ⁻¹)	1326 \pm 113	1366 \pm 306	288 \pm 58	421 \pm 134
Cl ⁻ (mg g ⁻¹)	1.49 \pm 0.22	3.84 \pm 0.35*	1.53 \pm 0.40	2.53 \pm 0.75
SO_4^{2-} (mg g ⁻¹)	0.68 \pm 0.25	0.48 \pm 0.12	3.28 \pm 1.30	2.40 \pm 0.34
NO_3^- (mg kg ⁻¹)	7 \pm 1	6 \pm 1	9 \pm 3	13 \pm 4
Oxalate (mg kg ⁻¹)	782 \pm 189	915 \pm 375	1604 \pm 515	1391 \pm 432
Malate (mg kg ⁻¹)	77 \pm 33	112 \pm 57	222 \pm 174	146 \pm 74
Citrate (mg kg ⁻¹)	13 \pm 8	48 \pm 54	270 \pm 81	280 \pm 97

* indicates significant difference from CK at $p<0.05$ with t-test.

Concentration of total free amino acid in young shoots of plants supplied with Cl was only 60% ($p=0.051$) that in the CK treatment. Concentrations of Thea, His and Tyr were significantly reduced whereas Ser was increased by Cl (Table 6). Concentrations of total and individual free amino acids (except Val) in fibrous roots were unaffected. However, the ratio of Thea to total free amino acid decreased significantly in both young shoots and fibrous root of plants receiving Cl.

Table 6. Free amino acids ($\mu\text{mol g}^{-1}$) in young shoots and fibrous roots of plants supplied with (+Cl) and without Cl (CK) (Experiment 3). Data are means \pm standard deviations ($n=4$).

Amino acid	Young shoots		Root	
	CK	+Cl	CK	+Cl
Thea	87.31 \pm 28.06	33.50 \pm 8.41*	278.94 \pm 65.75	232.19 \pm 24.67
Gln	59.00 \pm 21.04	47.92 \pm 15.24	200.96 \pm 70.47	259.00 \pm 100.71
Glu	29.06 \pm 6.93	25.15 \pm 4.05	16.17 \pm 4.77	16.70 \pm 4.88
Arg	24.80 \pm 23.42	6.53 \pm 3.98	42.77 \pm 35.93	38.84 \pm 22.76
Asp	8.78 \pm 1.80	7.44 \pm 1.82	12.30 \pm 5.05	14.32 \pm 3.23
Asn	0.86 \pm 0.31	0.75 \pm 0.15	13.62 \pm 1.13	32.24 \pm 19.86
Ala	2.69 \pm 0.92	2.93 \pm 1.35	2.70 \pm 1.39	3.48 \pm 2.44
GABA	1.05 \pm 0.25	0.81 \pm 0.31	4.10 \pm 0.44	5.52 \pm 1.52
Gly	1.52 \pm 0.89	1.62 \pm 0.38	0.83 \pm 0.17	0.91 \pm 0.03
His	2.05 \pm 0.36	0.71 \pm 0.16*	9.08 \pm 2.30	10.11 \pm 1.94
Ile	0.28 \pm 0.17	0.27 \pm 0.03	0.36 \pm 0.14	0.40 \pm 0.09
Leu	0.44 \pm 0.24	0.31 \pm 0.07	0.42 \pm 0.14	0.57 \pm 0.12
Lys	1.51 \pm 1.44	0.41 \pm 0.09	1.71 \pm 0.75	1.52 \pm 0.51
Met	0.05 \pm 0.09	0.15 \pm 0.12	0.31 \pm 0.24	0.46 \pm 0.19
Orn	n.d.	n.d.	2.93 \pm 0.39	5.12 \pm 2.22
Phe	0.56 \pm 0.16	0.50 \pm 0.06	0.43 \pm 0.08	0.51 \pm 0.17
Ser	2.44 \pm 0.77	3.78 \pm 0.41*	2.46 \pm 0.28	3.62 \pm 1.37
Thr	1.45 \pm 0.36	1.34 \pm 0.32	5.60 \pm 7.01	2.88 \pm 0.58
Try	0.43 \pm 0.14	0.27 \pm 0.06	0.78 \pm 0.29	0.68 \pm 0.48
Tyr	1.79 \pm 0.44	0.53 \pm 0.04*	1.01 \pm 0.01	1.30 \pm 0.31
Val	0.58 \pm 0.29	0.76 \pm 0.19	1.04 \pm 0.18	1.62 \pm 0.29*
Sum	226.65 \pm 71.46	135.67 \pm 29.09	598.51 \pm 196.92	631.98 \pm 102.89

n.d.: not detected. * indicates significant difference from CK at $p<0.05$ with t-test.

Discussion

Concentration of total free amino acid was increased by application of K_2SO_4 , a finding consistent with data from field experiments (Ruan et al., 1999). In contrast, application of KCl with an equal K amount as in the K_2SO_4 treatment had no influence (in 2001), or reduced (in 2002) concentration of total free amino acid compared to the control (without K application). The negative effect of KCl became more evident by direct comparison with K_2SO_4 and was observed even when growth of young shoots had not been significantly affected. These results confirm earlier findings (Ruan et al., 1998) and further showed that decrease in total free amino acid was principally caused by a reduced concentration of Thea, which was affected greater than any other free amino acids. The effect of KCl appeared to be quite

specific on the free amino acid (Thea). Other important quality-related components (i.e. polyphenols and caffeine) increased following K application either as KCl or K₂SO₄.

In this study, K was provided as KCl or K₂SO₄ at agronomically pertinent amounts as indicated by the yield response. In experiment 3, a low Cl concentration (equivalent to NH₄⁺) was applied, which was much smaller than those commonly used for salinity studies (e.g. 40~200 mmol L⁻¹). These experimental approaches were adopted to prevent induction of salt stress and/or osmotic stress. Analysis of elemental concentrations from Experiments 1 and 3 (Tables 1 and 5) and Experiment 2 (data not shown) revealed no indication of nutrient imbalance being induced by Cl. Their (except Cl) concentrations in plant tissue were hardly affected and could not account for the decrease of Thea concentration in young shoots. On the other hand, increasing absorption of Cl following Cl addition has been offset mainly by decreasing SO₄²⁻ concentration (Tables 1 and 5). The smaller pool of SO₄²⁻ in plant tissues might also result from decreasing application of S by substitution of K₂SO₄ with KCl. To maintain a sufficient S supply, ammonium sulphate was applied in the experiments. The S status in plants was further monitored by analyzing SO₄²⁻ concentration. Concentration of SO₄²⁻ in plants was not largely changed, though a smaller concentration was generally observed in Cl-treated plants (Tables 1 and 5). Nevertheless, in some cases KCl application, compared to the control, even increased concentration of SO₄²⁻ (Table 1), possibly due to improvement of plant growth. The measured values of SO₄²⁻ concentrations in leaves appeared to be well above critical levels suggested for many woody plants (80~100 mg kg⁻¹, Ul Haq and Carlson, 1993; and references therein). Supply of Cl significantly stimulated Cl accumulation in leaves, found to be consistent with a decrease of Thea in young shoots. The overall results described above suggest that decreased Thea concentration in young shoots following Cl application was most likely due to Cl accumulation in plants.

The mechanism underlining the negative effect of Cl on accumulation of free amino acids in young shoots has not been clarified (Ruan et al., 1998). Production and accumulation of free amino acids in tea plants depend on an adequate N supply (Chapter 3). Reduced concentrations of total free amino acid and Thea were thus firstly ascribed to an inhibition effect of Cl on N absorption and utilization. This appeared to be supported by the data of ¹⁵N labeling experiment showing N absorption decreased following Cl application. However, since Cl toxicity occurred in this experiment, the possibility that decrease of nutrient uptake was not confined to N but a general effect applicable to other nutrients as well, cannot be completely excluded. On the other hand, the effect of Cl has been specifically attributed to inhibited NO₃⁻ absorption and reduction (Cerezo et al, 1997; 1999; Lea-Cox and Syvertsen, 1993; Ruiz and Romero, 2001). Surprisingly, Cl application increased the concentration of NO₃⁻ in plant tissues (Figure 4). This finding suggests absorption of NO₃⁻ might not have been significantly inhibited and was likely a result of accumulation because of depressed reduction, which agrees with the previous observation showing that Cl reduces the activity of nitrate reductase (Ruan et al., 1998). To further verify this assumption, tea plants were grown with NH₄⁺ nutrition with an effective nitrification inhibitor (DMPP) to inhibit any potential nitrification (Padgett and Leonard, 1993). No detectable NO₃⁻ was found in the nutrient solution.

This, in combination with only trace amounts of NO_3^- found in plant tissue (Table 5) indicated a pure NH_4^+ nutrition was well maintained. Decrease of Thea concentration in young shoots observed under these conditions indicated that Cl likely imparted an inhibitory effect in addition to that on NO_3^- reduction. Results from the same experiment showed NH_4^+ uptake and its subsequent incorporation into Gln were probably not influenced by Cl since specific NH_4^+ absorption rate (per dry root base), GS activity and concentrations of Gln and Glu were all at similar levels between plants supplied with and without Cl. Furthermore, synthesis of Thea did not appear to be affected as there were similar Thea concentrations in fibrous roots. Thea is synthesized in roots and translocated via xylem to physiologically active organs such as young shoots where it accumulates or is hydrolyzed to glutamate and ethylamine by theanine hydrolase (Sasaoka et al., 1965; Takeo 1974; Tsushida, 1987; Tsushida and Takeo, 1985). Therefore, a reduced Thea concentration in young shoots but not in fibrous roots suggests translocation of Thea from roots to shoots and its further catabolism in young shoots might be influenced by Cl, inviting future investigation.

It has been generally accepted that the critical Cl toxicity concentrations are about 4-7 and 15-50 mg g^{-1} for Cl-sensitive and Cl-tolerant plant species, respectively (Marschner, 1995; Xu et al., 2000). In the present experiment, tea plants already suffered from Cl toxicity when Cl concentration in the mature leaves reached 8.5 mg g^{-1} . However, the content of chlorophyll in leaves and accumulation of free amino acids in young shoots had already been reduced when the Cl concentration reached approximately 4 mg g^{-1} , although plant growth had not been negatively affected yet. These data indicated that tea plants are sensitive to Cl and may be classified as a Cl sensitive plant species.

Although the present data and earlier work (Ruan et al., 1998) showed reduced concentration of free amino acids, in particular Thea, in young shoots of tea plants receiving Cl under controlled conditions, a number of field experiments conducted in China in the previous years did not indicate any large difference between application of KCl and K_2SO_4 (Ruan et al., unpublished data). Moreover, a recent study (Venkatesan and Ganapathy, 2004) and personal unpublished data showed increasing concentration of free amino acid in young shoots of tea plants receiving KCl fertilization in the fields. Such discrepancy might be attributed to several factors leading to different Cl absorption and accumulation in plants (especially in leaves). Firstly, various tea varieties were used for pot and field experiments and varietal difference in Cl absorption and tolerance might exist. Secondly, the availabilities of Cl to plants in these two experimental systems were greatly different. In fields, Cl-fertilizers are often applied in autumn as a basal dressing after the tea season. A major portion of Cl might have already been leached out of the root layer before intensive absorption occurred in March of the next year, as Cl^- is readily mobile in soil (Yang and Blanchar, 1996). Split applications of KCl make it possible that relatively small amounts of Cl were applied each time (Venkatesan and Ganapathy, 2004). In addition, in fields only a portion of the root system has immediate access to Cl-fertilizers, which are often applied in bands between two rows of tea bushes. Contrarily, in pot experiments the whole root system is exposed to Cl soon after application due to compacted root distribution in a small soil volume. Thirdly,

morphological differences of plant materials could play a crucial role in determining Cl absorption and concentration in leaves. Young plants of limited size are often used in pot experiments while in fields plant sizes of bushes are much larger. More importantly, shoot to root ratios for young tea plants (1:1) are largely different from those for tea bushes in fields (2~3:1) (Tea Research Institute, 1985). Since uptake of Cl from enriched mediums is likely a passive process (Marschner, 1995; Moya et al., 1999), absorption of Cl is therefore initially determined by the size of the root system while its concentration in leaves is basically a function of the size of leaf biomass (a dilution effect) and is likely modulated by shoot to root ratios (Moya et al., 1999). Consequently, it is expected the amounts of Cl taken up per unit root size in the field are relatively smaller because there is only partial access to applied Cl, and smaller concentrations in leaves due to more intensive dilution by greater leaf biomass and shoot to root ratios than in the case of young plants in pot experiments.

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Summary

The taste of tea may be characterized by astringency, bitterness, mellowness and slight sweetness. Free amino acids including the tea unique theanine, are the principal contributors to the tastes of freshness and mellowness of green tea. The polyphenols (mainly as catechins) are responsible for astringent and bitter tastes. Caffeine also contributes to bitterness of tea infusions. Therefore, free amino acids, polyphenols and caffeine are particularly important groups of compounds for taste and quality of green tea infusions. Their production and accumulation in the young shoots are thus of uppermost importance. In this study, the influence of nutrient supply on their accumulation in tea plants was investigated under controlled conditions to establish the functional relationship between nutrition, physiological processes and product quality of green tea.

The effect of N form and its interaction with root-zone pH on growth and nutrient uptake of tea plants was investigated in a nutrient solution experiment. Plants supplied with NO_3^- grew much slower than those receiving NH_4^+ or $\text{NH}_4^+\text{+NO}_3^-$ independent of pH treatments. These plants had significantly reduced concentrations of total N and free amino acids as well. Absorption of NO_3^- was considerably less than NH_4^+ and was strongly depressed when NH_4^+ was simultaneously supplied. The results indicated that tea plants were adapted to NH_4^+ as the sole N source due to their large capacity for NH_4^+ assimilation characterized by sharply increasing root glutamine synthetase activity and improving carbohydrate supply to the roots. Poor growth with NO_3^- was largely associated with inefficient absorption of this N source. Plant biomass production was the least at pH 6.0 but the highest at pH 5.0 regardless of N form supplied. Growth response to low root-zone pH varied with N forms, being reduced in NO_3^- supplied plants but not in those receiving NH_4^+ or $\text{NH}_4^+\text{+NO}_3^-$. Growth inhibition caused by an inappropriate root-zone pH was due partly to declining absorption of nitrogen.

Quality of green tea positively correlates with concentrations of free amino acids (especially theanine). Concentrations of free amino acids in young tea shoots were strongly affected by the N forms supplied in the nutrient solution, being considerably larger in NH_4^+ - than in NO_3^- -fed plants. This was firstly attributed to the larger absorption rate of NH_4^+ over NO_3^- . Furthermore, the relative allocation of absorbed N to the major free amino acids (particularly theanine and glutamine) was substantially increased by supplying plants with NH_4^+ , suggesting that NH_4^+ was more readily assimilated into theanine than NO_3^- . This is explained by the differences in enzyme activity, compartmental assimilation of different N forms and the availability of intermediates for synthesis of free amino acids. The concentration of caffeine was larger in plants receiving NH_4^+ - and $\text{NH}_4^+\text{+NO}_3^-$ - than in NO_3^- -fed plants whereas catechin concentrations were smaller in $\text{NH}_4^+\text{+NO}_3^-$ -supplied plants than in other plants. The results clearly indicated that only the supply of NH_4^+ led to larger concentrations of free amino acids, especially theanine, and caffeine. Root-zone pH did not influence concentrations of most free amino acids in the young shoots except theanine, which increased at pH 4.0 irrespective of N forms in the nutrient solution. This surprising observation, which contrasts with the reduced absorption of N by low root-zone pH, was attributed to an accumulation effect because at low pH, plant

growth was reduced more than N absorption.

Quality of the two major teas, black and green, is differentially influenced by concentrations of polyphenols, free amino acids, caffeine and their relative proportion in the young shoots. The plain quality of black tea positively correlates with contents of catechins, which serve as precursors for theaflavins and thearubigins. In contrast, quality green tea requires only appropriate concentration of polyphenols but high concentrations of free amino acids. Furthermore, the taste of green tea is also influenced by the relative proportion of individual free amino acids, each of which has the specific taste of sour, salty, sweet, bitter and umami (the Japanese word 'umami' means 'delicious' and represents the characteristic sensory property of monosodium glutamate). Free amino acids, caffeine and flavonoids are produced from primary or secondary metabolism. The effect of N status on primary and secondary metabolisms and concentrations of their metabolites in relation to the accumulation of major quality-related components were therefore investigated. Plants were supplied with varying N concentrations in the nutrient solution (N₁ 0.35, N₂ 0.75, N₃ 1.5 and N₄ 4.5 mmol L⁻¹). Concentrations of most catechins except (-)-epigallocatechin gallate (EGCG) in young shoots were reduced by the highest N supply. Biosynthesis of the catechins did not appear to be related to the concentration of free phenylalanine and activity of phenylalanine-ammonia lyase. Concentrations of total N, free amino acids, and caffeine as well as activity of phosphoenolpyruvate carboxylase and glutamine synthetase increased with N concentration in the nutrient solution. The decrease of catechins was linked to reduced glucose and fructose concentrations in both young shoots (sink) and recently matured leaves (source). These results suggest that more carbon resources were allocated to N metabolism, leading to a decrease in substrates available for formation of catechins in the young shoots. To illustrate relative changes of individual free amino acids, their ratios to the total concentration (sum of all free amino acids) were calculated and vector analysis performed. Increasing N concentration in the nutrient solution generally promoted accumulation of free amino acids. However, the concentration of theanine and its ratio to total free amino acid augmented greatly when the external N supply rose from N₁ to N₃ whereas its ratio decreased with further increase of external N due to a dilution effect. The ratios for arginine and glutamine to the total increased sharply with the highest N supply, indicating that relatively more absorbed N was allocated to synthesis of these amino acids. This is possibly caused by reduced carbohydrate concentrations thus, an imbalanced C : N status in the roots, which induced synthesis of amino acids with low C : N ratios to improve carbon economy.

There is little information on Cl nutrition in tea plants, i.e. its absorption and distribution, its effect on plant growth and product quality is available. Therefore, the absorption and effect of Cl on accumulation of free amino acids in young tea plants was investigated in pot experiments. In the first experiment, tea plants were grown in pots with soil and supplied with different levels of potassium as K₂SO₄ or KCl. Analysis of samples during a period of two years showed that concentrations of free amino acids in young shoots increased only when K₂SO₄ was supplied while they were unchanged in the first year or decreased in the second year when supplied with KCl at an equivalent K amount. The decrease of total free amino acids was mainly caused by reduced concentrations of theanine, which could not be accounted for by

the effect of Cl on the absorption of other nutrients and was more likely due to Cl accumulation in the plants. The absorption and impact of Cl on nitrogen uptake were further investigated in a second experiment in which soil-cultivated tea plants were provided with varying amounts of Cl as KCl supplemented with NH_4Cl . Application of Cl reduced yield of young shoots and induced foliage injury. The severity of leaf damage was related to concentration of Cl in the leaves. N uptake (measured with ^{15}N) of plants was reduced by Cl addition. NO_3^- accumulated in plant tissues possibly due to depressed reduction by Cl. To verify whether the decrease of free amino acids is simply a result of inhibition on NO_3^- assimilation, a third experiment was conducted cultivating tea plants with only NH_4^+ nutrition with Cl (at the same concentration as N) or without Cl. Similarly to the observation from the first experiment, concentrations of theanine and total free amino acid in young shoots were reduced by Cl. This result indicated that Cl likely has inhibitory effect in addition to that on NO_3^- reduction. However, the concentration of theanine in the roots, where it is synthesized, was unaffected. Total N concentrations in the roots and mature leaves, the specific absorption rate of NH_4^+ and the activity of glutamine synthetase in fibrous roots and young leaves were all not influenced by Cl. Based on the overall data, it is suggested that translocation of theanine from root to shoot and its further catabolism in the young shoots might be influenced by Cl. The results are discussed in comparison with those from field experiments, which did not show any significant effect of Cl from KCl application as compared to K_2SO_4 , in terms of different Cl absorption and accumulation in the plants between pot and field experiments.

In addition to their importance for quality, flavonoids, theanine and caffeine also possess beneficial functions to human health. The experimental results indicate that biosyntheses of these biologically active constituents are affected by nutritional status of plants. Therefore, it is feasible that nutrient availability may be manipulated to produce tea rich in these compounds.

Zusammenfassung

Der Geschmack von Tee lässt sich durch Adstringens, Bitterkeit, Weichheit und leichte Süße charakterisieren. Die freien Aminosäuren, die auch die teespezifische Aminosäure Theanin einschließt, sind die wichtigste Komponenten für die Frische und Weichheit von Grüntee. Die Polyphenole (überwiegend Catechine) sind verantwortlich für die Adstringens und den bitteren Geschmack. Koffein trägt ebenfalls zur Bitterkeit von Grünteeinfusionen bei. Somit sind die freien Aminosäuren, die Polyphenole und Koffein besonders wichtige Gruppen von Verbindungen, die den Geschmack und die Qualität von Grüntee bestimmen. Ihre Synthese und Anreicherung in jungen Triebspitzen sind daher von größter Bedeutung. In der vorliegenden Untersuchung wurde der Einfluss des Nährstoffangebots auf die Akkumulation qualitätsbestimmender Inhaltsstoffe unter kontrollierten Bedingungen untersucht, um funktionale Beziehungen zwischen der Nährstoffversorgung, den physiologischen Prozessen und der Produktqualität von Grüntee zu etablieren.

Der Einfluss der N-Form und der Interaktion zum pH-Wert im Wurzelbereich auf Wachstum und Nährstoffaufnahme bei Teepflanzen wurde in Nährlösungsversuchen untersucht. Pflanzen, die mit NO_3^- versorgt wurden, wuchsen wesentlich langsamer als solche die NH_4^+ oder $\text{NH}_4^+ + \text{NO}_3^-$ erhielten, unabhängig vom pH-Wert der Nährlösung. Die NO_3^- -ernährten Pflanzen wiesen signifikant geringere Gehalte an Gesamt-N und freien Aminosäuren auf. Die Absorption von NO_3^- war erheblich geringer als die von NH_4^+ und zudem stark vermindert, wenn NH_4^+ gleichzeitig angeboten wurde. Diese Ergebnisse zeigen, dass Teepflanzen durch ihre hohe Kapazität für die NH_4^+ -Assimilation, charakterisiert durch die starke Zunahme der Glutaminsynthetase-Aktivität der Wurzel und die verbesserte Kohlenhydratversorgung derselben an NH_4^+ als alleinige N-Quelle gut adaptiert sind. Das schlechte Wachstum mit NO_3^- war größtenteils auf die ineffiziente Absorption dieser N-Quelle zurückzuführen. Die Biomasseproduktion war unabhängig von der angebotenen N-Form bei pH 6.0 am geringsten, hingegen bei pH 5.0 am größten. Die Wachstumsreaktion bei niedrigem pH-Wert hing von der N-Form ab, denn das Wachstum war beim Angebot von NO_3^- vermindert, nicht jedoch beim Angebot von NH_4^+ oder $\text{NH}_4^+ + \text{NO}_3^-$. Diese Wachstumsdepression war zumindest teilweise eine Folge der geringeren N-Absorption bei niedrigen externen pH-Werten.

Die Qualität von Grüntee ist positiv korreliert mit dem Gehalt an freien Aminosäuren (insbesondere Theanin). Deren Konzentration in jungen Trieben war stark beeinflusst durch die in der Nährlösung angebotene N-Form, denn NH_4^+ -Ernährung führte zu drastisch erhöhten Gehalten verglichen mit NO_3^- -Ernährung. Dies war in erster Linie auf die größere Aufnahme von NH_4^+ im Vergleich zu NO_3^- zurückzuführen. Zudem war die relative Allokation des absorbierten N zur Synthese der dominierenden freien Aminosäuren (insbesondere Theanin und Glutamin) durch das Angebot von NH_4^+ deutlich erhöht, was darauf hindeutet, dass NH_4^+ rascher zu Theanin assimiliert wird als NO_3^- . Dies wird durch die Unterschiede in den bestimmten Enzymaktivitäten, der unterschiedlich kompartimentierten Assimilation der verschiedenen N-Form und der Verfügbarkeit der Intermediate für die Aminosäuresynthese unterstützt. Die Konzentration an

Koffein war beim Angebot von NH_4^+ - und von $\text{NH}_4^++\text{NO}_3^-$ höher als bei NO_3^- , während die Catechinkonzentration beim Angebot von $\text{NH}_4^++\text{NO}_3^-$ geringer war als bei den anderen Pflanzen. Diese Ergebnisse zeigen eindeutig, dass nur beim Angebot von NH_4^+ größere Konzentrationen an freien Aminosäuren, insbesondere Theanin, und Koffein erzielt werden. Der pH-Wert des Wurzelraums hatte keinen Einfluss auf die Konzentration der meisten freien Aminosäuren in jungen Sprossen mit Ausnahme von Theanin, dessen Gehalt bei pH 4.0 unabhängig von der angebotenen N-Form erhöht war. Dieses überraschende Ergebnis, welches mit der verminderten N-Absorption bei niedrigem externen pH-Wert kontrastiert, wurde auf einen Akkumulationseffekt zurückgeführt, da bei niedrigem pH-Wert das Pflanzenwachstum stärker beeinflusst war als die N-Absorption.

Die Qualität der wichtigsten Tees, Schwarz- und Grüntee, wird durch die Konzentrationen an Polyphenolen, freien Aminosäuren Koffein und ihre Verhältnisse zueinander in jungen Sprossen unterschiedlich beeinflusst. Die Qualität von Schwarztee korreliert positiv mit dem Gehalt an Catechinen, welche als Vorstufen von Theaflavinen und Thearubiginen dienen. Im Gegensatz dazu erfordert qualitativ hochwertiger Grüntee nur mäßige Polyphenolgehalte, aber hohe Gehalte an freien Aminosäuren. Zudem ist der Geschmack von Grüntee durch den relativen Anteil einzelner Aminosäuren zueinander bestimmt, die spezifische Geschmackskomponenten hervorrufen (sauer, salzig, süß, bitter sowie ‚umami‘ (dieses japanische Wort meint ‚wohlschmeckend‘ und bezeichnet die charakteristische Geschmacksempfindung von Natriumglutamat).

Freie Aminosäuren, Koffein und Flavonoide werden im Primär- und Sekundärstoffwechsel gebildet. Daher wurde der Einfluss der N-Versorgung auf den Primär- und Sekundärstoffwechsel und die Konzentration deren Metabolite in Beziehung auf die Akkumulation der wichtigsten wertgebenden Komponenten in Nährlösungskultur untersucht (N_1 0.35, N_2 0.75, N_3 1.5 und N_4 4.5 mM). Die Konzentrationen der meisten Catechine, mit Ausnahme des (-)-Epigallocatechin-gallats (EGCG) in jungen Sprossen war bei dem höchsten N-Angebot vermindert. Die Biosynthese der Catechine war offensichtlich nicht durch die Konzentration an freiem Phenylalanin oder die Aktivität der Phenylalanin-ammoniumlyase bestimmt. Die Konzentrationen an Gesamt-N, freien Aminosäuren und Koffein sowie die Aktivitäten der Phosphoenolpyruvat-Carboxylase und der Glutaminsynthetase wurden durch steigendes N-Angebot erhöht. Die Abnahme der Catechingehalte stand in Beziehung zu verminderten Gehalten an Glukose und Fruktose sowohl in jungen Sprossen (sink) als auch in gerade voll entwickelten Blättern (source). Diese Ergebnisse lassen vermuten, dass zusätzliche C-Skelette in den N-Metabolismus eingebracht wurden, was zu einer Limitierung der Substrate für die Bildung der Catechine in den jungen Sprossen führte. Um die relative Veränderung einzelner freier Aminosäuren zu illustrieren wurde der Anteil einzelner freier Aminosäuren an der Gesamtkonzentration (an freien Aminosäuren) berechnet und eine Vektoranalyse durchgeführt. Ein zunehmendes N-Angebot in der Nährlösung (von N_1 auf N_3) förderte generell die Akkumulation freier Aminosäuren. Allerdings erhöhte sich die Theaninkonzentration und deren Anteil am gesamten freien Aminosäurepool deutlich, wenn die externe N-Konzentration von N_1 auf N_3 angehoben wurde, während ihr Anteil aufgrund eines Verdünnungseffektes bei weiter steigendem

N-Angebot wiederum sank. Die Anteile von Arginin und Glutamin am gesamten freien Aminosäurepool erhöhten sich nur beim höchsten N-Angebot drastisch, was eine verstärkte Allokation des absorbierten N zur Synthese dieser Aminosäuren anzeigt. Dies ist möglicherweise durch eine verminderte Kohlenhydratversorgung verursacht, was eine Verschiebung des C:N-Gleichgewichtes der Wurzel zur Folge hat, die ihrerseits die Synthese von Aminosäuren mit engerem C:N-Verhältnis zur Verbesserung der C-Ökonomie fördert.

Chlor ist zwar ein essentielles Spurenelement, über die Bedeutung von Cl für die Ernährung der Teepflanze, insbesondere dessen Absorption und Verteilung, die Wirkung auf Pflanzenwachstum, Ertrag und Qualität von Grüntee ist jedoch wenig bekannt. Daher wurde die Absorption von Cl und der Einfluss von Cl auf die Akkumulation freier Aminosäuren in jungen Teepflanzen in Gefäßversuchen untersucht. Im ersten Experiment wurden die Teepflanzen in Boden kultiviert, der mit unterschiedlichen Mengen an K in Form von K_2SO_4 und KCl gedüngt war. Die Analyse von Proben über einen Zeitraum von zwei Jahren zeigte, dass die Konzentrationen an freien Aminosäuren in jungen Sprossen nur erhöht war, wenn K in Form von K_2SO_4 zugeführt wurde. Dem gegenüber blieben die Gehalte im ersten Jahr unverändert bzw. waren im zweiten Jahr vermindert, wenn äquivalente Mengen an K in Form von KCl gedüngt wurde. Die Abnahme des Gehalts an freien Aminosäuren war überwiegend auf eine verminderte Theaninkonzentration zurückzuführen, welche nicht auf den Einfluss von Cl auf die Aufnahme anderer Nährstoffe zurückgeführt werden konnte, sondern eher auf die Anreicherung von Cl selbst. Die Auswirkung von Cl auf die Absorption von Stickstoff wurde in einem zweiten Versuch betrachtet, in welchem im Boden kultivierte Teepflanzen mit unterschiedlichen Mengen an Cl aus KCl, und ergänzend aus NH_4Cl behandelt wurden. Die Cl-Applikation minderte den Ertrag an jungen Sprossen und verursachte Nekrosen der Blätter, und das Ausmaß der Blattschäden stand in Beziehung zur Cl-Konzentration der Blätter. Die N-Aufnahme (bestimmt mittels ^{15}N) der Pflanzen wurde durch die Cl-Zugabe vermindert, was aber vermutlich nicht auf N beschränkt war, sondern eine allgemeiner Effekt war, der auch auf andere Nährstoffe zutrifft, da der Wurzelstoffwechsel durch Cl-Toxizität beeinträchtigt war. Die NO_3^- -Akkumulation im Gewebe war möglicherweise auf eine durch Cl gehemmte Reduktion zurückzuführen. Im dritten Versuch wurden Teepflanzen mit NH_4^+ als alleiniger N-Quelle und ohne bzw. mit Cl (bei gleicher Konzentration wie NH_4^+) angezogen, um zu prüfen, ob die Abnahme der Gehalte an freien Aminosäuren lediglich das Ergebnis einer Hemmung der NO_3^- -Assimilation darstellt. Entsprechend der Beobachtungen des ersten Versuchs waren die Konzentrationen an Theanin und der Gesamtpool an freien Aminosäuren in jungen Sprossen durch das Cl-Angebot vermindert. Diese Ergebnisse zeigen, dass Cl zusätzlich zum Einfluss auf die NO_3^- -Reduktion höchstwahrscheinlich einen hemmenden Effekt hat. Allerdings war die Theaninkonzentration in der Wurzel, dem Ort der Theaninsynthese, unbeeinflusst. Die Konzentration an Gesamt-N in der Wurzel und in ausgewachsenen Blättern, die spezifische Aufnahmerate von NH_4^+ und die Aktivität der Glutaminsynthetase in fibrösen Wurzeln und jungen Blättern waren allesamt unbeeinflusst durch Cl. Aufgrund der zur Verfügung stehenden Daten insgesamt wird geschlussfolgert, dass die Translokation von Theanin von der Wurzel in den Spross und dessen weitere

Verstoffwechslung in jungen Sprossen durch Cl beeinflusst sein mag. Die Ergebnisse werden unter bezug auf Feldversuche, welche abweichend zum Gefäßversuch keinen signifikanten Cl-Effekt bei KCl-Anwendung im Sinne einer Absorption und Akkumulation von Cl in den Pflanzen zeigten, diskutiert.

Zusätzlich zu ihrer Bedeutung für die Qualität von Grüntee besitzen Flavonoide, Theanin und Koffein positive Wirkungen auf die menschliche Gesundheit. Die experimentellen Ergebnisse zeigen, dass die Biosynthese dieser bio-aktiven Substanzen durch die Nährstoffversorgung der Pflanzen beeinflusst wird, woraus folgt, dass es möglich ist durch gezielte Beeinflussung des Nährstoffangebots hohe Gehalte dieser Inhaltstoffe zu gewährleisten.

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