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Genotype, nitrogen fertility and sulphur availability interact to affect flavour in garlic (*Allium sativum* L.)

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SUMMARY
Experiments were carried out to investigate the influence of combined nitrogen (N) and sulphur (S) fertility on the organosulphur compound content of garlic bulbs, especially alliin, as a specific flavour quality trait related to the health-value of garlic. Three cultivars, 'Printanor', 'Morasol' and 'Messidrôme', were grown in the greenhouse and *in vitro*. Sulphur was increased in the macronutrients within the range 0 – 144 mg l^{-1}. Numerous combinations of both S and N were tested *in vitro*. In the greenhouse, increasing N levels, from 184.8 mg l^{-1} to 856.8 mg l^{-1}, were tested with a single high level of S (128 mg l^{-1}). Increasing S alone did not affect the growth or bulb weight of any garlic cultivar, but slightly increased the alliin content of bulbs. No symptoms of S deficiency were observed under greenhouse conditions, although the same garlic cultivars grown *in vitro* at 0 mg S l^{-1} clearly suffered, their size being greatly reduced. This suggests an important role for S contamination, such as atmospheric S, in the process of S absorption by garlic plants, which is discussed. Increasing N levels significantly accelerated garlic maturity and decreased bulb yield. The effect of N on the organosulphur compound content of bulbs was cultivar-dependent, but alliin accumulation was not enhanced. 'Printanor' and 'Morasol' had the highest alliin levels and were negatively influenced by increasing N, while a slight synergistic effect of S and N was observed on 'Messidrôme'. 'Messidrôme' also had the highest flavour potential when including precursors, and was more efficient in accumulating S into organosulphur compounds. It is therefore advisable to choose the most appropriate variety, and to adapt the N × S fertilisation regime according to the environment, in order to produce garlic with the highest health-value.

Garlic (*Allium sativum* L.) was among the earliest domesticated species of plants. Vegetative reproduction *via* cloves or aerial bulblets has been promoted over the centuries, which has given rise to vigorous plants with large bulbs. Nowadays, garlic is cropped worldwide and is of major economic importance, being traded and consumed in most countries (Takagi, 1990). Garlic has long been known for its health benefits. Numerous therapeutic properties attributed to garlic include anti-fungal, anti-bacterial, anti-viral, anti-thrombotic, anti-tumor, hypotensive, hypoglycemic and hypolipidemic activities (Augusti, 1996; Sato, 2000). More recently, its therapeutic value related to cardiovascular diseases, cholesterol metabolism, atherosclerosis (Kik *et al*., 2001; Collin, 2004) and cancer (Le Bon and Siess, 2000) has been reported. These benefits can be linked to the organosulphur compounds found in bulbs, particularly the alkyl and alkenyl cysteine sulphoxides (Block, 1985). Among these, S-allyl-L-cysteine sulphoxide, or alliin, is derived from related γ-glutamyl peptides, including γ-glutamyl-S-allyl-cysteine (GLUAlCs), and accumulates in high concentration in garlic. When a garlic bulb is cut or crushed, the enzyme alliinase (EC 4.4.1.4) is released and transforms alliin into diallyl thiosulphinate, or allicin, a volatile compound which produces the characteristic garlic odour and flavour (Block, 1985; Block *et al*., 1993; Randle and Lancaster, 2002). Trace, or low quantities of other alkyl cysteine sulphoxides can also be found in garlic. Isoalliin, the major flavour precursor found in onion, which derives from γ-glutamyl-S-(trans-1-propenyl)-L-cysteine (GLUPeCs or IsoGLUAlCs) is also present in garlic (Hughes *et al*., 2005). Alliin is the component often considered as a specific flavour quality trait related to the health-value of garlic. Recently, the anti-carcinogenic properties of garlic were improved, with a higher amount of alliin in garlic powders (Bergès *et al*., 2004).

Numerous studies have investigated how sulphur (S) nutrition affects flavour in onion, a close relative of garlic, by changing bulb pungency (Freeman and Mossadeghi, 1970; Hamilton *et al*., 1997; Randle, 1992; Randle and Bussard, 1993), as well as the biosynthetic pathway for organosulphur compounds (Randle *et al*., 1995). Other factors such as nitrogen (N) also affected pungency in onion and the biosynthetic pathway for

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organosulphur compounds (Gamiely *et al*., 1991; Randle, 2000). If S is therefore accepted as the primary factor influencing onion flavour, recent investigations have demonstrated that N × S interactions should be considered (Coolong and Randle, 2003).

Few reports exist on the effect of fertility on flavour changes in garlic. Increasing S levels have been tested over a limited range, which allowed an investigation of a range of flavour strengths much lower than that investigated in onion (Freeman and Mossadeghi, 1971). Nitrogen fertility has been also considered (Bertoni *et al*., 1998; 1992; Raysseguier, 1995), but not combined with S, or as a factor influencing bulb composition.

Our experiments were carried out under controlled conditions, in a greenhouse and *in vitro*. *In vitro* studies provide a useful model system to study the influence of environmental or trophic factors on *Allium* physiology (Kahane *et al*., 1997) and carbohydrate biochemistry (Kahane *et al*., 2001). Three garlic cultivars, from three distinct physiological groups (Messiaen *et al*., 1993), were tested, as differences in response among cultivars have been reported in onion (Randle *et al*., 1995) and garlic (Bertoni *et al*., 1988). Thus, the purpose of our work was to provide new information on the influence of S fertility and N × S interactions on the composition and concentration of organosulphur compounds, especially alliin, in garlic bulbs, in order to improve their growth conditions and quality traits related to the health-value of garlic.

MATERIALS AND METHODS

Plant material

Three French garlic cultivars were studied: 'Morasol' (MOR), a Mediterranean variety (Group I; Messiaen *et al*., 1993); 'Printanor' (PRI), a temperate variety (Group II); and 'Messidrôme' (MES), an Autumn variety (Group III). They were supplied as virus-free certified cloves by INRA, Avignon, France, and introduced and micropropagated *in vitro* as required for the experiments.

Experimental designs

Greenhouse experiments: Experiments were set up in an insect-proof greenhouse in Dijon, France, in 2002 and 2003. The three garlic cultivars were planted in boxes (60 cm × 40 cm × 30 cm; 12 cloves per box) filled with washed river sand on 1 March 2002 and 3 February 2003, respectively. Plants were grown under natural daylight and daylength. Night and day temperatures were set at 18°C and 26°C, respectively. Three boxes per treatment were planted for PRI and MOR, and one for MES, in 2002; while two boxes per treatment were planted for each variety in 2003.

Fifty ml d^{-1} to 100 ml d^{-1} of three fertilising solutions were supplied by drip irrigation. Deionised water was supplied during the weekends to leach salt residues. Water and fertilising solution rates were related to the stage of the plants (Raysseguier, 1995).

In 2002, S levels were varied in the macronutrients of the fertilising solutions at 0, 32 and 128 mg S l^{-1} to provide deficient, normal and high S treatments, which were distinguished by the symbols Sd, Sn and Sh, respectively. The Sn fertilising solution was composed of 779 mg l^{-1} $Ca(NO_3)_2$ $4H_2O$, 556 mg l^{-1} KNO_3, 73 mg l^{-1} $(NH_4)_2$ HPO_4, 246 mg l^{-1} $MgSO_4$ $7H_2O$, 1 ml l^{-1} Fe-EDTA and micronutrients (1.47 mg l^{-1} H_3BO_3, 1.85 mg l^{-1} $MnCl_2$, 1.04 mg l^{-1} $ZnSO_4$, 0.24 mg l^{-1} $CuSO_4$, 0.05 mg l^{-1} H_2MoO_4). The pH was adjusted to 5.8 with 1 M HCl solution. In the Sd solution, $MgCl_2$ was substituted for $MgSO_4$ in the macronutrients to provide an S-deficient environment (Randle, 1992). In the Sh solution, S was supplied using 200 mg l^{-1} K_2SO_4, 241 mg l^{-1} $CaSO_4$ $2H_2O$ and 357 mg l^{-1} $MgSO_4$ $7H_2O$ to balance K, Ca and Mg, and represented excessive S in the fertility programme.

In 2003, the interaction of N with S was investigated by increasing N in the form of NH_4NO_3 in the Sh solution. Three N levels were tested: normal (184.8 mg l^{-1}), high (520.8 mg l^{-1}) and very high (856.8 mg l^{-1}), resulting in N treatments referred to as Nn, Nh and Nh+.

Plant growth was assessed every 10 d by counting the numbers of green and senesced leaves, and measuring the diameter of the growing bulbs, as appropriate. The height of the longest green leaf was also measured in 2003. In each year, garlic was harvested when two-to-three leaves remained green. Whole plants were then weighed and the roots were washed and dried. After 1 week of drying at room temperature, bulbs were removed, and three-to-six bulbs per replicate were selected, weighed and prepared for chemical analysis.

In vitro *experiments*: Material was introduced *in vitro* and micropropagated following the cyclic multiplication previously reported for onion (Kahane *et al*., 1992). The temperature, sucrose, light spectrum, and light intensity required during the bulbing process had been investigated and optimised in preliminary experiments. Bulbing was induced by a period of cold (2 months at 3°C; 10 h photoperiod of white light) followed by bulb formation in a warm environment (22° – 24°C) with a 16 h photoperiod under white light (Universal Light fluorescent tubes; OSRAM SASU, Molsheim, France) plus far-red light (185W incandescent tubes; ARIC, Aubervilliers, France). Light intensity was controlled at 95 µmoles m^{-2} s^{-1} with a radiation sensor (LI-189 Quantum/Radiometer/Photometer; LI-COR, Lincoln, NE, USA). The standard medium was based on a modified Murashige and Skoog medium (1962), as previously described (Kahane *et al*., 1992), and enriched with sucrose to assist the bulbing process in garlic. This resulted in a mineral composition of 1.9 g l^{-1} KNO_3, 1.6 g l^{-1} NH_4NO_3, 440 mg l^{-1} $CaCl_2$ $2H_2O$, 170 mg l^{-1} KH_2PO_4 and 370 mg l^{-1} $MgSO_4$ $7H_2O$ for the macronutrients, and 6.2 mg l^{-1} H_3BO_3, 0.025 mg l^{-1} $CoCl_2$, 10.6 mg l^{-1} $ZnSO_4$, 25 µg l^{-1} $CuSO_4$, 22.3 mg l^{-1} $MnSO_4$, and 0.83 mg l^{-1} KCl for the micronutrients.

An experiment was conducted to establish the sufficiency ranges for S fertility under *in vitro* conditions. Increasing S levels by 3-fold over standard media was tested on PRI and MOR by adjusting the macronutrients with Na_2SO_4. Sulphur deficiency was tested on PRI by substituting $MgCl_2$ for $MgSO_4$ in the macronutrients. We then established three S treatments: deficient, normal and high, at 0, 48 and 144 mg S l^{-1}, respectively. They were distinguished by the symbols Sd, Sn and Sh, respectively, and applied to PRI, MOR and MES for testing. Twenty-four plants per treatment were used for PRI, while 16 plants per treatment were used for the other two varieties.

A second experiment was conducted to study the interaction of N and S *in vitro* using numerous combinations of S and N. Nitrogen was varied by adjusting the level of NH_4NO_3 in the medium. This resulted in four N levels distinguished by the symbols Nd, Nn, Nh and Nh+ representing deficient (544.1 mg N l^{-1}), normal (824.1 mg N l^{-1}), high (1384.1 mg N l^{-1}) and very high (1944.1 mg N l^{-1}) N levels, respectively. In addition, S was varied at the Sd, Sn and Sh levels previously described. Nine S × N media combinations were tested on PRI, to produce the following treatments: (1) Sn × Nn; (2) Sn × Nh; (3) Sn × Nh+; (4) Sh × Nd; (5) Sh × Nn; (6) Sh × Nh; (7) Sh × Nh+; (8) Sd × Nn; (9) Sd × Nh+. Due to limited numbers of explants, all but treatments (3) and (7) were tested on MOR, while MES was tested only against treatments (1), (2), (5) and (6). Plant numbers for PRI, MOR and MES were 48, 32 and 28 per treatment, respectively.

Plant growth was assessed *in vitro* every 10 d. As plants were grown in tubes (2.5 cm diameter; 15 cm high), several observations were possible. The developmental stage of each plant was assessed according to: vegetative stage, onset of bulbing, active bulbing, leaf desiccation, mature bulb, and mature plant with no bulb. The size of the plant from the basal plate to the tip of the highest green leaf, and the number of green leaves, were also measured during growth and development. Bulbs were harvested individually, as they reached maturity. After being separated from the medium, roots and leaves, bulbs were washed, and dried for 3 d at ambient temperature. They were then weighed, measured (height and diameter), their condition was recorded, and they were prepared for analysis of alliin and its peptides.

Analysis of organosulphur compounds

An ion-pairing method, using HPLC, was used to quantify S compounds from bulbs. This technique, without derivatisation, allows the quantification of allicin, alliin, isoalliin and their peptide intermediates [γ-glutamyl-S-allyl-cysteine (GLUAlCs) and γ-glutamyl-S-(trans-1-propenyl)-L-cysteine (GLUPeCs); Arnault *et al*., 2003]. This method has provided detailed information on S-metabolism and the S-status of a large number of garlic accessions cultivated under different climatic conditions (Kamenetsky *et al*., 2005).

Garlic bulbs from the greenhouse and *in vitro* experiments were crushed in a 90:10 (v/v) methanol: water mixture acidified with 0.05% orthophosphoric acid to stop enzymatic reactions. Three-to-five bulbs per replicate were sampled from the greenhouse experiment, while all harvested bulbs were used from the *in vitro* cultures (≥ 16 bulbs per treatment). The extracts were then diluted for HPLC analysis. The water was acidified with formic acid in a 1:1000 (v:v) ratio. Solvent to tissue ratios were 3:1 (v:w) (Kamenetsky *et al*., 2005).

Chromatographic separations were performed using a Waters 616 pump and DAD 996 diode-array detector (Waters Corporation, Milford, MA, USA). Compounds were separated on a 150 mm × 3 mm i.d. × 3 µm particle Hypurity Elite C18 column Thermo Quest, at 38°C (Thermo Hypersil Division, Keystone, Bellefonte, PA, USA). Detection was with an UV detector at 208 nm. Separation required an elution gradient with two solvents, A and B. Solvent A consisted of 20 mM sodium dihydrogen phosphate and 10 mM heptane sulphonic acid (pH 2.1). Solvent B consisted of 50% A and 50% acetonitrile. The flow-rate of the eluant was 0.4 ml min^{-1}. The gradient run was programmed as follows: 100% A : 0% B for 4.9 min; 70% A : 30% B over the next 20 min; 46% A : 54% B for 1 min; 0% A : 100% B for 4 min; then held at 100% A for the next 10 min. Data acquisition was performed using Millenium software (Waters Corporation).

Analysis of total sulphur in plants

Total S analyses to monitor S absorption by plants, were performed in the 2002 greenhouse experiment at Warwick HRI. *In vitro* plants were not analysed due to a lack of sufficient tissue. Three plants per replicate were harvested twice for PRI (at the onset of bulbing, and at plant maturity); while MES plants were sampled only at maturity. Harvested plants were divided into leaf, bulb and root tissues. Each fresh tissue was weighed and freeze-dried until no further weight loss was observed, ground to a fine powder using a ball mill (Glen Creston, Stanmore, UK) and stored at –40°C until needed.

Total S was extracted by a modified version of Method 72 (ADAS, 1986) as follows: a 1 g sample of dried garlic powder was ashed at 450°C in the presence of 10 ml 2.7 M $Mg(NO_3)_2$, then boiled for 2 min in 10 ml 36% (w/w) conc. HCl and subsequently diluted in 25:1 (v/v) distilled water to dissolve the SO_4^{2-}. The extract was filtered to remove insoluble material and analysed on a JYHoriba Ultima 2 Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES; Jobin Yvon Ltd., Stanmore, Middlesex, UK), according to the manufacturer's instructions. The principle was to dissolve, in hydrochloric acid, the total S present as sulphate in the residue after destruction of the organic plant material.

Statistical analysis

Results from the *in vitro* experiments were expressed as means and standard deviations from two-to-four replicates per treatment. For the greenhouse experiments, ANOVA ($P = 0.05$) was used to establish statistical differences with SYSTAT 10 software using an unbalanced design.

RESULTS

Plant growth and bulb fresh weights (FW)

Sulphur nutrition did not affect the growth of garlic plants in the greenhouse, and no symptoms of S-deficiency were noticed in any of the cultivars. In accordance with the growth observations, no influence of S, but a strong cultivar effect ($P < 0.05$) was observed for bulb FW. 'Printanor' had the largest bulbs (mean FW = 22.8 ± 0.5 g), MES had the smallest bulbs (mean FW = 16.8 ± 2.2 g) and MOR was intermediate (mean FW = 19.1 ± 1.4 g). No interaction was found between cultivar and S nutrition.

As in the greenhouse, increasing S in the medium did not affect the growth of plantlets *in vitro*. Plantlets grown under the Sn and Sh regimes did not show any differences. However, a strong influence of S-deficiency was observed *in vitro* under Sd conditions. 'Printanor'

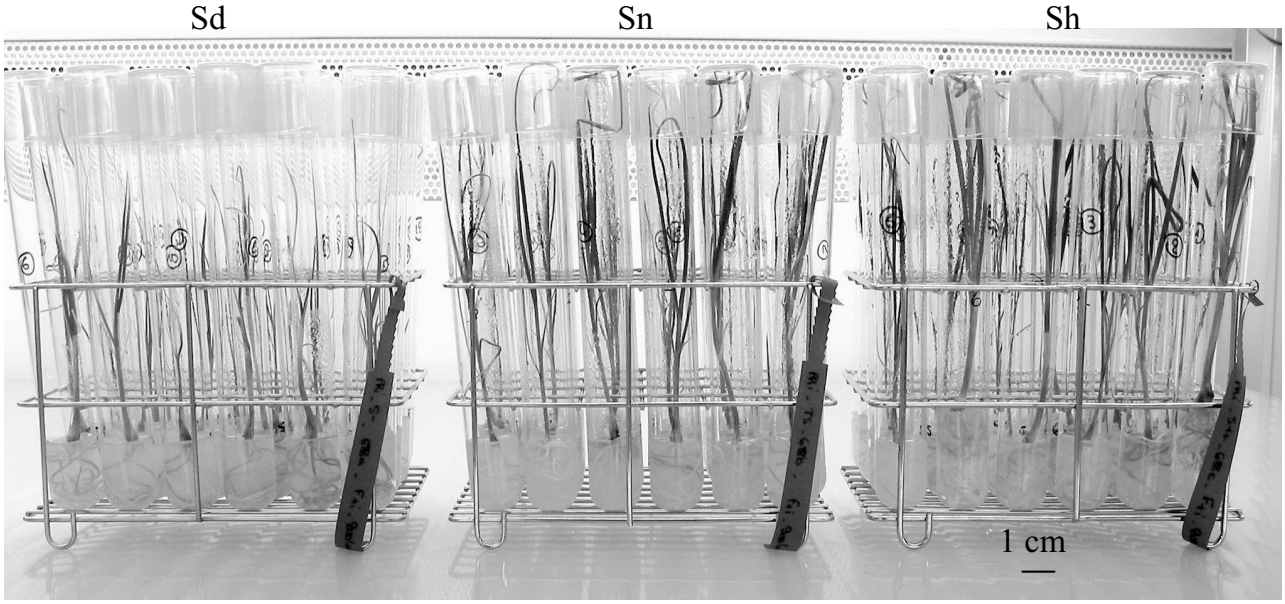

FIG. 1
'Printanor' plants after 47 d growing *in vitro* under three sulphur treatments: deficient (0 mg l^{-1}), normal (48 mg l^{-1}) and high (144 mg l^{-1}), distinguished by the symbols Sd, Sn and Sh, from left to right, respectively.

and MOR plantlets grown under Sd remained much smaller than the plantlets grown under other S conditions, with size decreases of 38.0% and 17.6%, respectively, compared to plants grown under normal conditions (Figure 1). The bulbing process was also affected, with numerous green and soft bulbs, considered abnormal, being produced under Sd, compared to the firm bulbs harvested under normal conditions. The percentage of normal bulbs harvested per treatment was recorded and used to distinguish the effect of S-deficiency on *in vitro* growth. It showed that bulbing in PRI was particularly sensitive to S-deficiency. Between both experiments *in vitro*, PRI produced 31% fewer normal bulbs under Sd × Nn compared to plants grown under Sn × Nn, while MOR was less affected under the same conditions (12% fewer normal bulbs). MES was not tested under Sd.

The normal level of nitrogen (Nn) favoured better foliage development under our greenhouse conditions, compared to the higher nitrogen levels of Nh and Nh+. Increasing N fertility, combined with a high S supply, accelerated maturity in all garlic varieties. This affected plant size and bulb yield, particularly for MOR and MES, as plants were harvested earlier (Table I). Under Nh, mean bulb weights decreased by 5.9% and 21.3% for MOR and MES, respectively. This effect was even stronger under Nh+, where mean bulb weights decreased by 34.9% and 39.3%, respectively. No variety × N interaction was observed.

Increasing N supply *in vitro* did not significantly accelerate maturity. Only a slight negative influence on foliar development was observed for PRI grown under the highest N supply, at both S levels, as the plantlets grown under Sn × Nh+ and Sh × Nh+ were slightly smaller (Table II). As a consequence, increasing N levels negatively affected mean bulb weight. This condition was not tested on MOR or MES, so the generality of this tendency could not be confirmed. Conversely, although decreasing N did not seem to affect plant growth *in vitro*, a slight increase was observed in the mean bulb weight of plants grown under Sh × Nd, especially PRI plants (Table II).

Sulphur × N treatments did affect PRI bulbing *in vitro*. Indeed, the percentage of PRI plants that produced normal bulbs increased to ≥ 80% when N was increased under both Sn and Sh conditions. A dramatic negative effect of increasing N was observed under S-deficiency (Sd), as less than 30% of PRI plants produced normal bulbs under Sd × Nh+ conditions.

TABLE I
Harvest data (± standard errors) in garlic cultivars 'Printanor' (PRI), 'Morasol' (MOR) and 'Messidrôme' (MES) grown in a greenhouse in 2003 under increasing nitrogen (N) and at high sulphur supply (Sh)

Garlic variety	N treatment	Harvest date	Maximum size of the longest green leaf (cm)	Mean weight of harvested bulbs (g)
PRI	Nn*	20/6	102.0 ± 4.0	14.67 ± 2.78
MOR	Nn	06/6	88.7 ± 6.4	14.60 ± 2.85
MES	Nn	26/5	88.1 ± 7.5	15.77 ± 3.95
Mean			92.9 a[1]	15.01 a
PRI	Nh	10/6	80.3 ± 6.3	15.11 ± 2.48
MOR	Nh	05/6	79.8 ± 5.7	13.73 ± 3.06
MES	Nh	26/5	72.8 ± 7.4	12.41 ± 2.82
Mean			77.6 b	13.75 ab
PRI	Nh+	05/6	77.5 ± 5.5	13.76 ± 3.77
MOR	Nh+	26/5	75.0 ± 4.4	9.50 ± 3.02
MES	Nh+	16/5	69.8 ± 6.7	9.57 ± 4.23
Mean			74.1 b	10.94 b

*See text for codes Nn, Nh and Nh+.
[1]Values followed by different lower-case letters differ significantly ($P < 0.05$).

TABLE II
Harvest data (± standard errors) in garlic cultivars 'Printanor' (PRI), 'Morasol' (MOR) and 'Messidrôme' (MES) grown in vitro *under various nitrogen (N) and sulphur (S) treatments*

Variety	S × N Treatment*	Maximum size of plantlets green part (cm)	Mean weight of normal harvested bulbs (g)	% Plants giving a normal bulb
PRI	Sd × Nn	13.3 ± 3.4	0.391 ± 0.069	61.7
	Sd × Nh+	12.6 ± 3.4	0.364 ± 0.067	29.2
	Sn × Nn	18.9 ± 1.9	0.330 ± 0.064	77.1
	Sn × Nh	18.7 ± 2.2	0.341 ± 0.119	83.3
	Sn × Nh+	16.8 ± 2.6	0.259 ± 0.095	83.3
	Sh × Nd	19.2 ± 1.9	0.433 ± 0.112	79.2
	Sh × Nn	19.1 ± 1.5	0.336 ± 0.104	72.9
	Sh × Nh	19.5 ± 1.8	0.380 ± 0.152	81.3
	Sh × Nh+	18.3 ± 2.5	0.274 ± 0.063	85.1
MOR	Sd × Nn	15.5 ± 3.7	0.232 ± 0.059	84.8
	Sd × Nh+	15.3 ± 2.9	0.206 ± 0.096	85.7
	Sn × Nn	18.7 ± 3.1	0.310 ± 0.082	93.8
	Sn × Nh	18.0 ± 2.7	0.303 ± 0.136	87.5
	Sh × Nd	18.6 ± 2.4	0.361 ± 0.136	93.3
	Sh × Nn	17.9 ± 2.2	0.310 ± 0.112	76.7
	Sh × Nh	17.5 ± 3.6	0.317 ± 0.106	90.0
MES	Sn × Nn	19.5 ± 1.3	0.281 ± 0.115	96.2
	Sn × Nh	17.6 ± 3.4	0.243 ± 0.085	100.0
	Sh × Nn	17.9 ± 2.8	0.221 ± 0.106	100.0
	Sh × Nh	18.4 ± 4.1	0.252 ± 0.089	96.2

*See text for codes Sd, Sn and Sh; and Nd, Nh and Nh+.

'Morasol' was similarly affected when grown under high S supply, but bulbing could be slightly improved by increasing N under Sh. However, bulbing in MES was particularly insensitive to mineral conditions. Almost 100% of plants of this variety produced normal bulbs under all conditions tested (Table II).

Total S contents

Sulphur accumulation in PRI and MES grown in the greenhouse in 2002 depended on the stage of development, tissue type and S fertility level (Table III). A significant effect of S-deficiency was observed on total bulb S content, which decreased strongly under Sd conditions for both varieties at any date. However, total bulb S contents did not increase significantly between Sn and Sh. Sulphur accumulation in bulbs did not differ significantly between cultivars.

Sulphur accumulation in leaves differed between cultivars, as it was affected by S-deficiency in PRI but not in MES, and was strongly affected by the stage of development. Sulphur accumulation in leaves decreased strongly at maturity, confirming the mobilisation of S from leaves to the bulb during the bulbing process (Table III). Roots were strong sinks for S at the onset of bulbing, especially under the highest S fertility treatment, suggesting that roots were the limiting factor in metabolising S at this stage. However, at maturity, bulbs were the strongest sink among the plant tissues analysed (Table III).

Organosulphur compounds

Analyses allowed for the quantification of alliin and the peptides, GLUAlCs and GLUPeCs (Figure 2). When grown under Sn conditions in the greenhouse, in 2002, PRI accumulated more alliin (107.8 nmol mg^{-1} FW) than either MOR (83.4 nmol alliin mg^{-1} FW) or MES (73.9 nmol alliin mg^{-1} FW) (Figure 2A). 'Printanor' and MOR also accumulated more alliin than MES when grown *in vitro* under the same S treatment (Figure 2B). However, in comparison with the greenhouse, alliin accumulation was substantially lower *in vitro*, while its biosynthetic precursors were greatly favoured in all cultivars. This was especially evident in both MOR and MES, which also showed more accumulation of alliin precursors than PRI when grown in the greenhouse (Figure 2A, B).

A significant interaction between garlic variety and S treatment was obtained for the concentrations of alliin and its precursors for plants grown in the greenhouse (Figure 3A). Although alliin concentrations generally increased with increasing S, the effect was only significant for MES ($P = 0.05$). GLUAlCs and GLUPeCs levels in MES bulbs were significantly lower under Sd, while no significant influence of S nutrition was observed in PRI and MOR.

In vitro, S-deficiency had a dramatic effect on the levels of S compounds in bulbs, and a strong and significant increasing effect of S levels was observed in PRI (Figure 3B).

TABLE III
Total sulphur (S)-content (± standard errors) analysed in freeze-dried samples from leaves, roots and bulbs of 'Printanor' (PRI) and from leaves and bulbs of 'Messidrôme' (MES) garlic grown in a greenhouse in 2002

Garlic Variety	Stage of development	Tissue	S content (in mg g^{-1} tissue)		
			Sd	Sn	Sh*
PRI†	Onset of bulbing	leaf	5.66 ± 0.14	7.10 ± 0.17	6.56 ± 0.49
		bulb	3.17 ± 0.31	5.39 ± 1.05	4.14 ± 0.14
		root	7.94 ± 0.40	10.95 ± 0.93	12.26 ± 2.49
PRI	Maturity	leaf	2.08 ± 0.46	3.33 ± 0.49	4.55 ± 1.34
		bulb	6.27 ± 0.87	7.88 ± 1.41	8.44 ± 0.76
		root	5.43 ± 0.08	6.31 ± 0.78	6.02 ± 1.09
MES	Maturity	leaf	2.50 ± 0.38	4.27 ± 1.84	3.46 ± 0.41
		bulb	5.36 ± 0.59	7.78 ± 2.07	8.30 ± 2.12

†Two stages were analysed for 'Printanor' (onset of bulbing and maturity).
*See text for codes Sd, Sn and Sh.

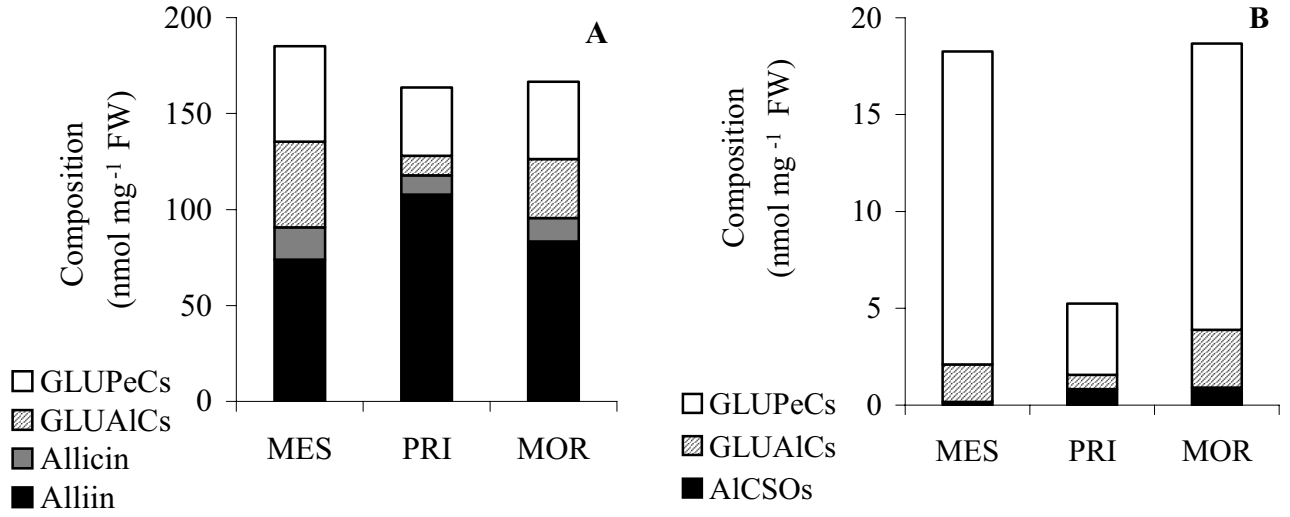

FIG. 2
Concentrations (in nmol mg^{-1} FW) of alkyl cysteine sulphoxides (AlCSOs), or alliin, allicin and the peptide precursors [GLUAlCs, γ-glutamyl-S-allyl-L-cysteine; and GLUPeCs, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine] in garlic bulbs of varieties 'Messidrôme' (MES), 'Printanor' (PRI) and 'Morasol' (MOR) grown under normal sulphur fertilisation at 32 mg S l^{-1} in a greenhouse in 2002 (Panel A), or *in vitro* at 48 mg S l^{-1} (Panel B).

The effect of N was also cultivar-dependent. Increasing N significantly decreased alliin contents in PRI bulbs grown in the greenhouse under Sh in 2003 (Figure 4). A significant decrease was also observed for MES under Nh, while no further effect was observed under Nh+. No significant differences were found for MOR across N levels (Figure 4). N-fertility generally had little effect on γ-glutamyl peptide contents. Only MES showed a positive response, inverse to that on alliin accumulation, to increasing N-fertility (Figure 4).

For plants grown *in vitro* under Sh, a similar cultivar-dependent effect of N could be observed. Increasing N, from Nn to Nh+, significantly decreased the alliin content of PRI only (Figure 5A–C). Contrary to what was observed in the greenhouse, the three varieties showed γ-glutamyl peptide content responses to N fertility. However, two different tendencies were observed: PRI and MOR showed a decreasing response to N above the normal level, Nn (Figure 5A, B); while in MES the response was to increase levels of γ-glutamyl peptides (Figure 5C).

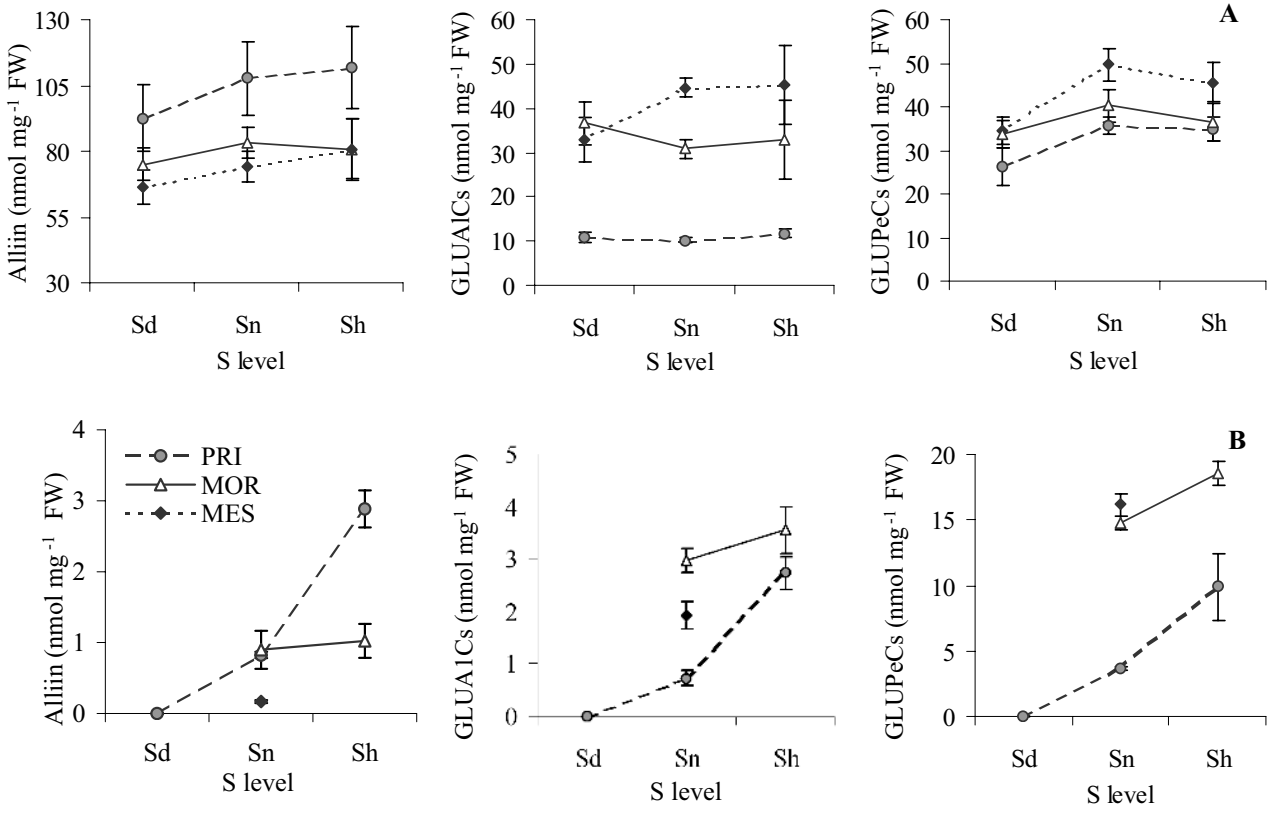

FIG. 3
Concentrations (in nmol mg^{-1} FW) of alliin and the peptide precursors [GLUAlCs, γ-glutamyl-S-allyl-L-cysteine; and GLUPeCs, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine] in garlic bulbs of varieties 'Messidrôme' (MES), 'Printanor' (PRI) and 'Morasol' (MOR) grown in 2002 in a greenhouse (Panel A) or *in vitro* (Panel B) under deficient, normal and high S levels, distinguished by the symbols Sd, Sn and Sh, respectively.

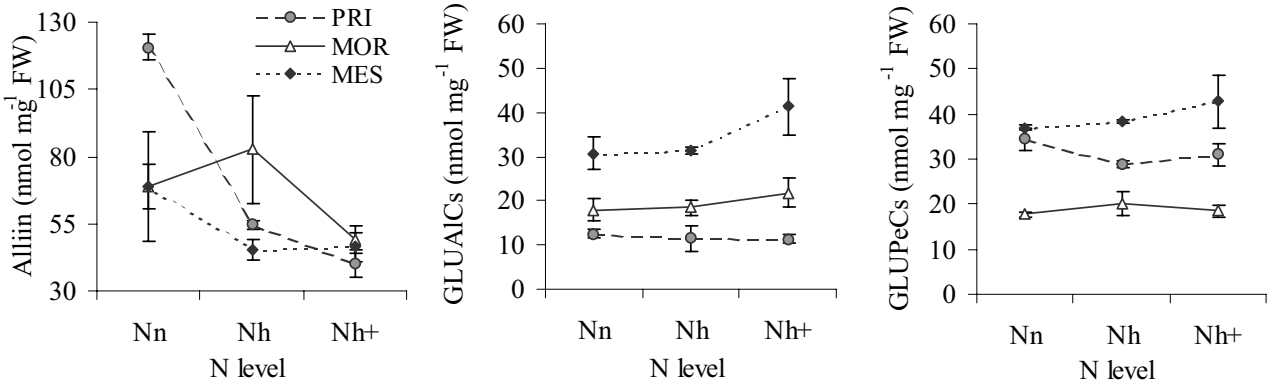

Fig. 4
Concentrations (in nmol mg^{-1} FW) of alliin and the peptide precursors [GLUAlCs, γ-glutamyl-S-allyl-L-cysteine; and GLUPeCs, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine] in garlic bulbs of varieties 'Messidrôme' (MES), 'Printanor' (PRI) and 'Morasol' (MOR) grown in a greenhouse in 2003 at 128 mg S l^{-1} under three nitrogen (N) levels: normal, high and very high, distinguished by the symbols Nn, Nh and Nh+, respectively.

Combinations of N with different S levels, and N-deficiency, were tested *in vitro* only. Almost no organosulphur compounds were found in PRI and MOR plants grown under Sd at any N level (e.g., Nn or Nh+; Figure 5A, B). For plants grown under Sn, a cultivar-dependent effect, similar to that observed for plants grown under Sh, was observed. 'Printanor' and MOR plants grown under N-deficiency and Sh showed decreased levels of alliin and GLUAlCs compared to Nn. The GLUPeCs response was reversed (Figure 5A, B).

DISCUSSION

Our experiments investigated unique combination of S and N in garlic, and their effects on the organosulphur content of bulbs related to flavour. We increased S fertility to levels which had been approached only once previously in studies on onion, but with no flavour measurements (Hamilton *et al*., 1997). In contrast, the effects on bulb pungency in both garlic (Freeman and Mossadeghi, 1971) and onion (Randle *et al*., 1995) were examined only within a range similar to our Sd-Sn levels. Similar work on organosulphur compounds was reported in onion, but not in garlic (Randle *et al*., 1995). In onion, S-fertility changed the levels of S-alk(en)yl-L-cysteine sulphoxides (AlCSOs) and their intermediates in bulbs grown in the greenhouse, with a cultivar-dependent effect (Randle *et al*., 1995). A change in organosulphur compound composition was also observed, but no effect of over-fertilisation with S was evident within the range tested.

Our work in the greenhouse confirmed the general effect of increasing S-fertility on the alliin content of garlic bulbs, and a cultivar-dependent effect. However, despite the wide range of S fertility used in the greenhouse, the effect on garlic flavour compounds was less than expected, suggesting that over-fertilisation did not result in the metabolism of organosulphur compounds in garlic plants. High levels of total S measured before bulbing in PRI at 128 mg S l^{-1} confirmed that S was absorbed by roots, but suggested that part of the S was stored in the vacuoles of cells in the form of sulphate, instead of being used through the AlCSOs pathway (Randle, 2001). Our results also suggest that MES was more efficient at accumulating S for flavour than the other two varieties.

In the greenhouse experiment, we did not observe any S-deficiency symptoms in the garlic cultivars tested, as previously described in onion plants (Chatterjee *et al*., 1999; Freeman and Mossadeghi, 1970; Randle *et al*., 1995). This confirmed previous investigations in garlic (Freeman and Mossadeghi, 1971), but also suggested that garlic could compensate for S-deficiency in mineral nutrition by using another supply of S, such as atmospheric S (De Cormis, 1968; Kühn and Faller, 1970). No symptoms of S-deficiency were observed in wild onion (*A. vineale*) when grown under deficient S-levels (Freeman and Mossadeghi, 1971), which suggests this effect could be species-dependent.

Contrary to our greenhouse experiments, we observed that the same garlic cultivars, grown *in vitro*, suffered when grown in an S-deficient medium. Sulphur was supplied in the micronutrients used in both experimental designs, based on the hypothesis that these very low quantities would not influence S-metabolism in plants grown under Sd. However, we can assume that less gaseous exchange occurred *in vitro*. Thus, the main difference in S-supply between the two experimental designs appears to arise from S contamination and suggests an important role for atmospheric S in the process of S absorption by garlic plants.

Recent work has confirmed that onion plants can use atmospheric S as their sole S-source for growth, and that hydrogen sulphide (H_2S) absorbed through the leaves was metabolised into flavour precursors (Dürenkampf and De Kok, 2004). Previous studies on garlic suggested that plants could compensate for a deficiency in S-fertility by absorption of atmospheric S (Freeman and Mossadeghi, 1971; Raysseguier, 1995). Although our experiments do not provide any direct quantitative validation of this hypothesis, the design of the *in vitro* experiment provides new information that supports it, and suggests that plants grown *in vitro* cannot access the same supply of S for their metabolic requirements as plants grown under greenhouse conditions.

Although *in vitro* culture provides a successful model for studies on plant physiology and carbohydrate metabolism in *Allium* (Kahane *et al*., 1997; 2001), the validity of this model system for studies on S-metabolism is questioned. Indeed, alliin accumulation levels were substantially lower and γ-glutamyl peptides accumulation, especially GLUPeCs, much higher in

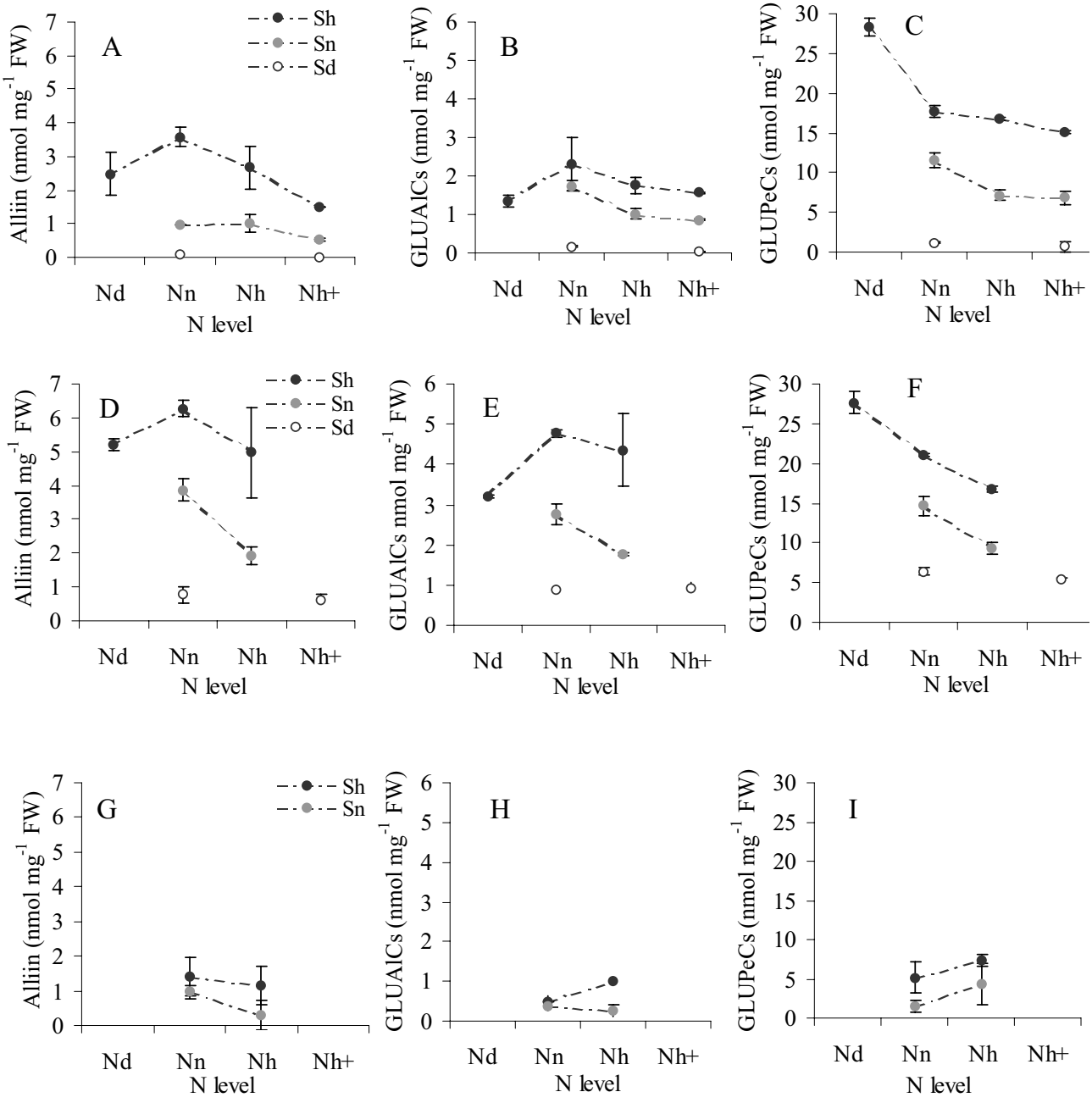

FIG. 5
Concentrations (in nmol mg^{-1} FW) of alliin and the peptide precursors [GLUAlCs, γ-glutamyl-S-allyl-L-cysteine; and GLUPeCs, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine] in garlic bulbs of varieties 'Printanor' (PRI, Panels A–C), 'Morasol' (MOR, Panels D–F), and 'Messidrôme' (MES, Panels G–I) grown *in vitro* under various S × N combinations. Three S levels: deficient (0 mg l^{-1}), normal (48 mg l^{-1}) and high (144 mg l^{-1}), distinguished by the symbols Sd, Sn and Sh, respectively, were combined with four levels of N, from deficient to very high, distinguished by the symbols Nd, Nn, Nh and Nh+, respectively.

plants grown *in vitro* than in a greenhouse, suggesting an effect of growing conditions.

GLUPeCs was also found in higher quantities than expected in garlic in the greenhouse, and isoalliin derived from this γ-glutamyl peptide is well-known to have a minor role in garlic flavour (Lawson, 1996). Other studies using the same method to analyse organosulphur compounds reported similarly high levels in many different garlic cultivars grown under two different climatic conditions (Kamenetski *et al.*, 2005), which suggests an involvement of GLUPeCs in alliin biosynthesis. However, despite recent studies into the biosynthetic pathway of alliin in garlic (Hughes *et al.*, 2005), the role of GLUPeCs is still unknown.

The high level of GLUPeCs found in garlic grown *in vitro* raises a question as to the role of this peptide in S-metabolism under conditions of S-deficiency. Previous work reported that flavour precursors are a strong sink for bulb S under conditions of low S supply (Lancaster *et al.*, 1998). This contrasts with the high proportion of S-peptides found *in vitro*. Recent work suggests that S-peptides are formed in high amounts and are not remobilised into alliin during exposure to H_2S (Dürenkampf and De Kok, 2004). These authors indicated that additional studies were needed to

understand the impact of H_2S on the biosynthetic pathway of γ-glutamyl peptides and alliin, which agrees with our observations.

Some similarities were found between our *in vitro* and greenhouse conditions. The influence of cultivar on S-composition and S-concentration in bulbs was observed under both conditions, which supports the value of *in vitro* studies for modelling genotype responses. The Spring varieties, PRI and MOR, showed high alliin contents, while the Winter variety (MES) had the highest flavour potential when considering levels of alliin and γ-glutamyl peptides as well. However, the health-value of the γ-glutamyl peptides is unknown. Similar results were also observed under both growing conditions on cultivar-dependent responses to increasing N. Again, MES showed a different behaviour, which suggests a difference in S metabolism among cultivars.

In our experiments, increasing N levels did not enhance the alliin content of bulbs. In fact, higher N strongly decreased the alliin contents of MOR, when grown *in vitro* under normal S supply, and of PRI under greenhouse conditions. Increasing N also decreased peptide levels in the Spring varieties, as previously observed on onion (Randle, 2000). A different response was observed with the Winter variety, MES, especially related to the γ-glutamyl peptides. Here, a slight synergistic effect on precursor levels was observed with increasing S × N. However, additional research is required to confirm this, due to the lower number of treatments tested on MES.

To improve the health-value of garlic bulb, genetic factors should be considered as well as the interaction between S and N levels. If we take into account the possible supply of S through leaves, by absorption of H_2S, culture conditions could also have a strong influence. Our results also question the role of precursors, especially GLUPeCs, in S-metabolism in garlic plants, which warrants further investigation.

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