Storage time and temperature affects volatile organic compound profile, alliinase activity and postharvest quality of garlic

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ABSTRACT

Garlic (Allium sativum L.) has a long history of use as a culinary seasoning and source of health-promoting compounds. In particular organosulphur compounds derived from the action of alliinase on alliin are of interest for their antimicrobial action. Due to the seasonal nature of the garlic harvest, long-term storage is required to ensure year-round supply of high-quality bulbs. However, quality is known to deteriorate throughout storage, and storage regimes are aimed at maintaining culinary, not biochemical quality, posing challenges for biotech firms extracting high value products, such as alliinase, from garlic. Storage typically involves extended periods of up to 9 months at -1.5 °C. Here, quality parameters (disease incidence and moisture content) as well as biotechnological quality parameters (alliinase yield and activity) were measured, and correlated with gene expression and volatile organic compound (VOC) profiles comparing storage at -1.5 °C and 22 °C. The aim is to develop potential molecular markers for garlic quality assessment. Alliinase activity fell in the first 6 months of storage, with garlic stored at -1.5 °C losing more activity, however 22 °C stored garlic suffered higher spoilage after 12 months storage. Alliinase activity loss was not proportional to gene transcript levels, suggesting post-translational control. A total of 150 VOCs were detected across all samples using thermal desorption gas chromatography, time of flight mass spectrometry of intact garlic bulbs, the most abundant of which were organosulphur compounds. Storage temperature significantly affected the whole VOC profile and discrete profiles were detected from garlic cold-stored for different time periods. Using weighted correlation network analysis 17 VOCs were identified that correlated with storage time, six VOCs that were indicative for storage temperature and four VOCs (azulene, octanal, o-Xylene and 4-methylhexadecane) were significantly associated with alliinase activity.

1. Introduction

Garlic (Allium sativum) is an important commercial food crop, harvested for its subterranean storage bulbs, and a plant of considerable interest in the biotechnology industry, with 28 million tonnes produced worldwide in 2017 (FAO, 2017). Besides our culinary interest in garlic, it has been used for over 4000 years as a medicinal plant, with some of the earliest references being from Pliny the Elder in Historia Naturalis, where 61 remedies involving garlic are described (Block, 2010). More recently, benefits of garlic on the cardiovascular system (Banerjee and Maulik, 2002; Ackermann et al., 2001; Gebhardt, 1993), in vitro antimicrobial properties (Harris et al., 2001; Reiter et al., 2017) and potential to reduce the risk of, or inhibit, cancer have been reported (Santhosha et al., 2013; Luo et al., 2016; Dorant et al., 1993). These properties are predominantly attributed to organosulphur compounds derived from the enzymatic action of alliinase on the cysteine sulphoxide alliin, producing allicin (Cavallito and Bailey, 1944; Ankri and Mirelman, 1999). Specifically, ajoene and related organosulphur compounds are being explored as novel antimicrobials for healthcare challenges, including antimicrobial-resistant lung and wound infections, through their action as inhibitors of quorum sensing in bacterial biofilms (Silva et al., 2018).

Alliin, a stable, non-volatile cysteine sulphoxide (CSO) is primarily synthesised in the leaves and translocated to the bulb where it is concentrated in vesicles within the cytoplasm of mesophyll cells in the cloves (Lawson, 1996). Accumulation of alliin and other CSOs is
multifactorial, varying according to genotype, locality, light quality and sulphur fertilisation (Montano et al., 2011; Huchette et al., 2004). Two biosynthetic pathways have been proposed for the synthesis of CSOs, firstly the alkylation of glutathione by γ-glutamyl peptides to yield S-alkyl cysteine sulphoxides and secondly, thioalkylation of serine and subsequent oxidation to cysteine sulphoxide (Jones et al., 2007). The serine pathway relies on cysteine synthase to convert serine to biosynthetic pathways have been proposed for the synthesis of CSOs, identified in garlic (Kamenetsky et al., 2015). In one study alliin increased to over twice its original concentration when garlic was stored for 83 d at ambient temperature (Bloom et al., 2011). In contrast, Hughes et al. (2006) found alliin remaining largely unchanged after 6 months of cold storage and Ichikawa et al. (2006) reported no significant changes from pre-storage levels in garlic stored at –3 °C or 23 °C across 9 time points over 150 d storage. Ichikawa et al. (2006) did, however, report a significant increase in allin concentration at 90, 120 and 150 d compared to pre-storage levels in the same garlic cultivar when stored at 4 °C. Differences may be therefore ascribed to temperature or cultivar effects.

Aliinase is highly abundant within garlic tissues and constitutes approximately 10 % of the total cell protein (Eltom and Feldberg, 1994). In bulbs, alliinase is localised in the vacuoles of the bundle sheath cells, surrounding the phloem vessels (Eltom and Feldberg, 1994). Due to their different sub-cellular localisation, alliinase cannot interact with alliin until there is mechanical damage to the bulb (Yamazaki et al., 2002). Alliinase is a dimeric glycoprotein which cleaves carbon-sulphur bonds and has a MW of 51.5 kDa per subunit and contains 6 % carbohydrate (Rabinkov et al., 1994). It employs a single pyridoxal-5-phosphate (PSP) molecule as a co-factor. During the growing period, alliinase protein is also found to a lesser degree in leaves and roots, but root alliinase has low amino acid sequence homology to leaf or bulb alliinase (Rabinkov et al., 1994). Alliinase mRNA is present in both leaves and bulbs at all growth stages and persists for as long as there are living cells (Smeets et al., 1997). Whilst alliinase is highly abundant and its mRNA constitutively expressed in garlic, the enzyme itself is thermolabile, denatured by allicin or by pH below 3.0 (Jansen et al., 1989; Blania and Spangenberg, 1991). Alliinase gene expression did not change significantly over a 6 month experiment on bulbs stored at different temperatures (Cho et al., 2012). Alliinase is encoded by a multigene family that has not been fully characterised, however, when 45 full-length alliinase amplicons from one cultivar of garlic were cloned, nine sequence variants were identified (Cavagnaro et al., 2003; Ovesná et al., 2015). Furthermore, the variability in the alliinase gene family indicated the presence of up to 14 sequence variants.

Allicin (S-prop-2-en-1-yl 2-propene-1-sulphinothioate) is a bioactive and very reactive molecule which, depending on the reaction conditions, rapidly yields further volatile organosulphur compounds (Block, 1992). For example, if kept at 20 °C for 20 h, allicin mainly yields diallyl disulphide (DADS, 66 %), diallyl sulphide (DAS, 14 %), diallyl trisulphide (9 %) whereas short term heat treatment results in 2-vinyl-4H-I, 3-dithiin and 3-vinyl-4H-I,3-dithiin, and ajoene is formed in larger proportions during maceration at room temperature (Block, 1985; Lawson and Gardner, 2005). Many of these products are also bioactive and of commercial interest and consequently the control of alliinase activity, precursors, and reaction conditions are key for the production of these organosulphur compounds from garlic (Ilie et al., 2011; Amazigo, 2006; Jones et al., 2007, Neem pers. com.)

Whilst current farming and storage practices ensure bulbs are free from disease, sprouting and damage, biochemical quality control is not routinely performed throughout the storage period. Bulbs are harvested in late summer through to autumn and must then be stored to ensure year round supply. They are stored commercially in warehouses at marginally sub-zero temperatures, around –2 °C, for up to 9 months to promote dormancy and prolong shelf life (Volk et al., 2004; Ito et al., 2020). However, it is not clear whether this storage regime is optimal for the maintenance of alliinase activity over time, posing challenges for biotechnology firms extracting alliinase or allin-derived organosulphur compounds from garlic. There is limited research on alliinase maintenance in stored garlic. Post-harvest storage conditions have been shown to affect alliinase activity in onion (Uddin and MacTavish, 2003), although this study was performed over a relatively short storage period of 9 weeks.

Here garlic was stored for 12 months at 22 °C and at -1.5 °C to assess effects on alliinase activity during post-harvest storage, and to assess whether changes in VOC profiles could be used as markers for garlic quality. Weighted correlation network analysis was used to identify subsets of VOCs that may be useful markers for assessing temperature of storage and changes in alliinase activity.

2. Materials and methods

2.1. Plant material

Garlic bulbs (Allium sativum var. Altanero), were sourced from Coopaman, 45 Las Pedroñeras, Cuenca, Spain. Bulbs were of Category I quality, having been inspected for disease and damage, and 60 – 65 mm in diameter. Bulbs were sourced after harvest, drying and processing at the Coopaman facility, prior to cold storage.

VOC collection was performed on whole, undamaged bulbs and all other measurements were performed after clove separation and only on healthy cloves. These were defined as cloves free from visible disease, excessive damage or severe desiccation. Sprouting cloves were considered healthy if the storage parenchyma was acceptably firm to the touch and not heavily discoloured and the sprout did not exceed 1 cm in length. Any cloves which did not meet these standards were counted, weighed then discarded prior to physiological measurements and alliinase extraction.

2.2. Garlic storage

Bulbs were stored together in single, well-ventilated containers in the dark at -1.5 °C ± 0.6 °C (cold storage) and 22 °C ± 3.5 °C (ambient storage) for a total of 12 months after arrival at Cardiff. Before storage (Time 0) and after 3, 6 and 12 months of storage, nine bulbs were sampled from each treatment. Temperature and humidity were monitored continuously using a data logger (THC-4, InkBird Tech. Co, Shenzhen, China). Relative humidity levels remained at 65 % ± 10 % in both storage conditions.

2.3. VOC sample collection

Three bulbs were selected at random and placed into a Nalophan bag (46 × 56 cm, Lakeland Ltd., Windermere, UK) and sealed using a food bag storage clip. A 0.5 mL Eppendorf tube with its tip cut off was inserted into the corner of the sampling bag by cutting off the corner of the bag and securing the cap with an elastic band. This served as a sampling port for the TD tubes (Tenax TA/Sulphicarb inert coated SafeLok TD Tubes, Markes International, Llantrisant, Wales, UK). After an equilibration time of 2 h, 7.5 L of headspace was sampled through the TD tube at a rate of 60 mL min-1 using a ‘SpeediVac’ High Vacuum Pump (Edwards High Vacuum Ltd, Crawley, UK), with tubes connected via a Q-Max Tube Holder (Supelco, Bellefonte, PA, USA) and flow rate calibrated with a GAP METER flow meter (GAP, Croydon, UK). Three replicates, each of three bulbs, were collected for each storage condition at each sampling point. Blanks of air were sampled from the laboratory in an identical fashion, without the addition of garlic bulbs.

2.4. Alliinase enzyme extraction

After sampling VOCs, the three bulbs of garlic were weighed and carefully separated into cloves. The outer sheath, scape and basal plate...
were discarded and the remainder was re-weighed. Cloves were inspected for disease and any showing signs of decay were counted, weighed then discarded. Of the remaining healthy cloves, a sub-sample of 50 g was taken at random and blended for 15 s in 150 mL chilled H2O and ice with a Philips HR1361 hand blender (Phillips Electronics Co. Ltd, Guildford, UK). Beakers were kept on ice during extraction. After 30 min on ice, the garlic suspension was passed through a handheld sieve lined with four layers of paper towel to remove large particulate matter and the pulp squeezed to release all free liquid. Celite Hyflo SuperCel filter aid (2.5 g, Fisher Scientific, Hampton, NH, USA) was added and the mixture agitated with a magnetic stirrer (Heidolph MR Hei-Standard, Schwabach, Germany) for 5 min. The mixture was transferred to 50 mL Falcon tubes and centrifuged at 3000 g for 10 min at 4 °C in a Sigma 3–16KL centrifuge (Sigma, Gillingham, Dorset, UK) and the pellet discarded. The pH of the solution was then lowered to pH 4 using 3 M acetic acid, added dropwise whilst stirring with a magnetic stirrer and measuring the pH with a pH 510 pH/mV/°C meter (Eutech Instruments Pte., Singapore), causing the enzyme to precipitate. The mixture was then re-centrifuged at 3000 g for 15 min at 4 °C and the supernatant discarded. The pellet was frozen, freeze dried for 72 h in a VirTis BenchTop Pro freeze drier (SP Scientific, Gardiner, NY, USA) and then re-centrifuged at 3000 g for 2 min, 5 °C min−1 to 280 °C followed immediately by a post-run temperature of 300 °C for 5 min. The BenchTOF-dx mass spectrometer (Almsco International, Blue Ash, OH, USA) was operated with an ion source temperature of 275 °C, transfer line temperature of 200 °C and a mass range of 35–650 m z−1. A retention time standard (C8–C20, Sigma Aldrich, Gillingham, Dorset, UK) was prepared by injection of 1 μL of the standard directly into an open-ended TD tube and analysed identically to the samples. GC-MS data were processed in ChemStation (E.02.01.1177; Agilent Technologies Inc., Didcot, UK) then deconvoluted and integrated with AMDIS (NIST, 2014), using a retention-indexed custom mass spectral library. MS spectra from deconvolution were searched against the NIST 2014 library (Mikaia et al., 2014). Compounds scoring > 80 % in forward and backward fit for their MS spectra and having a retention index (RI) falling within a range of ± 15 of literature values (Beaulieu and Grimm, 2001) were included as putatively identified compound in the custom library. Compounds that scored > 80 % in forward and backward fit for their MS spectra and showed no RI match were added as chemical class (e.g. alkane) and compounds that were consistently found but showed no MS spectral fit or RI match were added as ‘unknown’ the custom library. Only compounds present in the custom library were integrated and subjected to the subsequent data processing steps and statistical analysis. Compounds which were not present in all three replicates of at least one experimental group were excluded, along with compounds known to be common contaminants. Compounds present in all blank tubes at levels exceeding one tenth of the average abundance of samples were also excluded.

2.9. RNA extraction and purification

RNA extraction was according to Jordon-Thaden et al. (2015), with minor modifications. Samples were ground in a pestle and mortar under liquid nitrogen to a fine, homogenous powder and stored in 1 g aliquots at -80 °C until used. CTAB extraction buffer contained 2 % (w/v) hexadechltrimethylammonium bromide (CTAB), 1 % (w/v) polyvinyl pyrrolidone (PVP), 200 mM Tris–HCl buffer pH 8, and 1.4 M NaCl. To 1 mL of buffer, 2 μL 1-β-Mercaptoethanol (BME) was added prior to the extraction.

Ground garlic clove (100 μg) was added to a chilled 1.5 mL Eppendorf tube containing 900 μL of CTAB buffer and BME. Samples were vortexed immediately for 2 min and incubated at 55 °C for 8 min, this was repeated once and then samples were centrifuged at 13,000 g for 5 min at 20 °C (Sigma 3–16KL, Sigma, Gillingham, Dorset, UK). The supernatant (750 μL) was transferred to a chilled 1.5 mL Eppendorf tube and 750 μL of 24:1 chloroform:isoamyl alcohol was added, the phases were mixed by inversion and centrifuged at 13,000 g for 10 min at 4 °C. The aqueous phase was transferred to a 2 mL Eppendorf tube and 40 μL sarkosyl, 200 μL DH2O and 750 μL Tri-reagent (Invitrogen) were added and mixed by inversion. Samples were then centrifuged at 13,000 g for 10 min at 4 °C and the aqueous phase transferred to a 1.5 mL Eppendorf tube. Chloroform (200 μL) was added, before vortexing for 15 s, incubating at room temperature for 10 min and centrifuging at 13,000 g for or sporulation.

2.8. TD-GC-TOF-MS analysis

TD tubes were desorbed using a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK) using the following settings: tube desorption: 5 min at 100 °C, followed by 5 min at 280 °C, with a trap flow of 40 mL min−1, trap desorption and transfer to 300 °C for 5 min after heating at 20 °C s−1 starting from 25 °C, with a split flow of 5 mL min−1 into the GC (7890A; Agilent Technologies, Inc., Didcot, UK). VOCs were separated over a 60 m, 0.32 mm ID, 0.5 μm film thickness Rxi-5 ms (Restek, Bellefonte, PA, USA) at 2 mL min−1 constant flow of helium. The following temperature program was used: initial temperature 4 °C for 2 min, 5 °C min−1 to 280 °C followed immediately by a post-run temperature of 300 °C for 5 min. The BenchTOF-dx mass spectrometer (Almsco International, Blue Ash, OH, USA) was operated with an ion source temperature of 275 °C, transfer line temperature of 200 °C and a mass range of 35–650 m z−1. A retention time standard (C8–C20, Sigma Aldrich, Gillingham, Dorset, UK) was prepared by injection of 1 μL of the standard directly into an open-ended TD tube and analysed identically to the samples.

Alliinase activity was determined spectrophotometrically through the reaction between (4-mercaptopyrudine (4-MP, λmax = 324 nm) and allicin, forming 4-allylmercapthiopyridine, which has no absorbance in this spectral region (Miron et al., 2002). UV–vis measurements were performed in an Agilent Technologies (Didcot, UK) Cary 60 UV–vis spectrophotometer. Reaction mixtures were made individually and immediately prior to use and consisted of: 100 μL of 1 mM 4-MP (Fisher Scientific, Hampton, NH, USA), 500 μL of 0.1 M pH 7 potassium phosphate buffer, 10 μL of 2 mM pyridoxal-5-phosphate (Fisher Scientific, Hampton, NH, USA), 100 μL of 20 mM EDTA (Fisher Scientific, Hampton, NH, USA), 10 μL of 1 M alliin (Neem Biotech Ltd, Abertillery, Wales, UK) and 280 μL dH2O. Alliinase extract solution, consisting of 1 μL alliinase extract suspended in 500 μL of 0.1 M pH 7 phosphate buffer, was added to start the reaction. The mixture was vortexed for 10 s and absorption was recorded 30 s after mixing and every 10 s thereafter for 2 min. Alliinase activity was calculated using a standard curve of 20–100 μL of 1 mM 4-MP and a reagent blank.

Diseased cloves were discarded pre-extraction. An attempt was made separately to assess alliinase activity of diseased cloves, but no activity could be detected.

2.6. Bulb moisture content

Five cloves of garlic were selected at random from each replicate of each sample. Cloves were weighed to 5 decimal places using Mettler AMSO precision scales (Mettler Toledo Instruments, Leicester, UK) before being bisected longitudinally and dried in an electric oven at 80 °C for 7 d and reweighed.

2.7. Disease assessment

The number and weight of diseased cloves was recorded for all bulbs. A clove was described as diseased if it displayed signs of fungal attack, i.e. a region of discoloration, softening of tissues, mycelial growth and/or sporulation.

R.A. Ludlow et al.
15 min at 4 °C. The aqueous phase was transferred to an Eppendorf tube and this step was repeated if the sample did not look clear and colourless. RNA was precipitated with 750 μL of isopropanol (Fisher, Hampton, NH, USA), for 10 min at RT and pelleted by centrifugation at 13,000 g for 20 min at 4 °C. The pellet was washed in 75 % EtOH, dried and then resuspended in 20 μL dH₂O. Concentration and quality of the RNA was checked with a nanodrop spectrophotometer and by electrophoresis of 500 ng on a 1 % agarose gel.

2.10. DNAse and cDNA synthesis

RNA extract (2000 ng) was diluted to 16 μL with nuclease free water and, 2 μL RQ1 10x DNAse buffer (Promega, Southportm, UK) and 2 μL RQ1 DNase were added. The sample was incubated for 30 min at 37 °C. Samples were tested via PCR with UBQ primers (5′-AAGCCAGATGAGGCAAGA-3′, 5′-GCACTCCCTCCATCTC-3′, 153 nt amplicon, Mitrová et al., 2018) followed by gel electrophoresis to ensure removal of the DNA remaining in the sample, after which 2 μL RQ1 DNase STOP solution was added.

cDNA synthesis was performed using M-MLV RNase H- Reverse Transcriptase (Promega, Southportm, UK). To 20 μL of DNase treated RNA, 1 μL of 500 μg mL⁻¹ Oligo(dt) was added and incubated in a thermal cycler at 70 °C for 10 min then chilled on ice for 10 min. First strand buffer (6 μL of 5 x), 1.5 μL 10 mM dNTPs and 0.6 μL RNasin (25 μg) were added and incubated at 42 °C for 2 min. M-MLV reverse transcriptase (1 μL) was added, the mixture was incubated at 42 °C for 60 min, and inactivated by heating to 70 °C for 15 min.

2.11. PCR

Alliinase (AsAll) was amplified using AsAllF and AsAllR primers (5′-TGCTCATGCCCCCTTTATCA-3′, 5′-TTGATACGTGATGCGAGA-3′, 153 nt amplicon), cysteine synthase (AsCysK) was amplified using AsCysKF and AsCysKR primers (ATAGCAGCTGCGAAGGGTTA, 5′-ATTCTCTCCGCTTTTGAAT-3′, 152 nt amplicon) and normalised to garlic ubiquitin as a housekeeping gene (AsUBQ: Mitrová et al., 2018).

PCRs were carried out in 25 μL reactions (1 μL template cDNA, 0.5 μL 10 mM dNTPs, 5 μL Green Buffer (Promega, Southportm, UK), 1.5 μL 25 mM MgCl₂, 0.5 μL 10 μM primers, 0.125 μL Taq polymerase (Promega, Southportm, UK) and 15.9 μL dH₂O in an Applied Biosystems Veriti thermal cycler. The initial incubation was 5 min at 95 °C; followed by 35 x (45 s at 95°C; 45 s at 58.5 °C; 30 s at 72 °C) and 5 min at 72 °C.

2.12. qPCR

qPCR was performed using qPCRBIO SyGreen Blue Mix Lo-ROX ready mix (PCR Biosystems, London, UK) in a Roche Light Cycler 96 real time PCR system (Roche, Mannheim, Germany). The program consisted of an incubation at 95 °C for 2 min; 40 cycles of 3 step amplification 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and a melting curve. PCR was performed in 20 μL reactions consisting of 10 μL qPCRBIO SyGreen Blue Mix Lo-ROX (2X) (PCR Biosystems, London, UK), 0.8 μL 10 μM primers, cDNA (40 ng) made to 20 μL with nuclease free water. Reactions were run in technical triplicates from three biological replicates and included negative controls. Analysis was based on the ΔΔCt method by comparison to AsUBQ (Livak and Schmittgen, 2001).

2.13. Statistical analysis

Physiological and enzymatic measurements were performed in triplicate and analysed using GLM models in the R statistical program V3.4.2 (R Core Team, 2019). Time and temperature were used as predictors, with a gaussian error family and ‘identity’ link functions. Predictors were treated as categorical variables and interactions were analysed with a TukeyHSD post-hoc test. Pairwise significance for graphs were calculated using least significant differences (LSD) in the “agricolae” package in the R statistical program V3.4.2 (Steel et al., 1997; R Core Team, 2019).

Integrated signals of each VOC were normalised to total area in each sample and missing values were replaced with an abundance 1/5th of the minimum detected across all compounds and samples. Finally, data were square root transformed to reduce bias towards highly abundant compounds. Permutational multivariate analysis of variance (PerMANOVA) was performed to identify differences in the VOC profile associated with experimental parameters using the “vegan” package in R (Oksanen et al., 2019; Kindt and Coe, 2005). Ordination plots were generated via canonical analysis of principal coordinates discriminant analysis (CAPFullscrm, package “BiodiversityR”) and 95 % confidence ellipses were fitted (Anderson and Willis, 2003). Compounds significantly associated with temperature or time of storage were identified with weighted correlation network analysis (WCNA) using the WCNA package in R (Langfelder and Horvath, 2008). WCNAs were run with a soft power threshold of 6 and a minimum module size of 12.

3. Results

3.1. Disease incidence was greatest after 12 months of storage at 22 °C

The incidence of disease within samples increased sharply after 12 months of storage at 22 °C, with significant differences to all other time points and temperature treatments (GLM, P < 0.001). There were no statistically significant differences in disease rate between garlic stored at either temperature for the first 6 months of storage, nor did they differ from the baseline disease rates found at pre-storage (time 0). There was no significant increase in disease rate from pre-storage to 12 months of storage garlic at -1.5 °C (Fig. 1).

3.2. Moisture content remained constant throughout storage

Throughout storage, the moisture content of garlic remained constant, with no significant changes garlic stored at either 22 °C or-1.5 °C (ANOVA, Ptime = 0.074, Ptemp = 0.673, Ptime×temp = 0.737). The average moisture content of cloves was 64.2 % and ranged from 59.2 % to 68.0 % (Supplementary Table S1).

3.3. Alliinase extract yield fell with time under cold storage

Alliinase extract yields increased significantly after 3 months at -1.5 °C, before falling back to pre-storage yields at 6 months and significantly decreasing below pre-storage yields at 12 months (LSD, Fig. 2). Garlic stored at 22 °C retained an alliinase extract yield which did not differ

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**Fig. 1.** Proportion of diseased garlic cloves stored at 22 °C (ambient) and -1.5 °C (cold) for 0, 3, 6 and 12 months. Letters indicate statistically different values between the different temperatures and time points, based on GLM and LSD, alpha = 0.05, N = 3, error bars = SD.
3.4. Alliinase activity fell during the first 6 months of storage

Alliinase activity decreased significantly from pre-storage levels over time at both storage temperatures (GLM, \( p < 0.001 \), Fig. 3). Activity decreased significantly more in garlic stored at -1.5 °C throughout the first 6 months of storage (GLM, \( p < 0.001 \)) after 3 months of storage, alliinase activity was significantly lower in garlic stored at -1.5 °C, while the loss in activity at 22 °C was not statically significant. After 6 months there was significant loss of activity at both storage temperatures but the magnitude of activity loss at 22 °C was significantly less compared to storage at -1.5 °C (53.0 % loss vs. 84.8 % loss compared to pre-storage activity). However, after 12 months storage at 22 °C the garlic had decayed completely, and no enzyme activity was detectable while garlic stored at -1.5 °C retained 21.4 % of pre-storage enzyme activity.

3.5. Alliinase and cysteine synthase gene expression remained relatively stable during storage

Alliinase gene expression did not change significantly at any storage time point from pre-storage levels. However, when stored at -1.5 °C, expression increased significantly from month 6–12 (Fig. 4a). In contrast, cysteine synthase expression was maintained at a similar rate throughout storage and did not rise between 6 and 12 months of storage at -1.5 °C (Fig. 4b).

3.6. Composition of the VOC profile

A total of 150 compounds were putatively identified by comparison of mass spectra to NIST library and retention index data (Supplementary Table S2). Major chemical groups in the VOC profile of garlic included alkane (48), aromatic (16), alcohol (13), aldehyde (13), organosulphur (13), terpene (13), and cycloalkane (11).

Whilst just 13 of the 150 VOCs were organosulphur compounds, they included the most abundant compounds. Diallyl disulphide was the single most abundant VOC, followed by diallyl sulphide and alllyl methyl sulphide, accounting for an average of 29.0 %, 10.7 % and 5.1 % respectively of the total abundance across all samples (Supplementary Table S3).

3.7. Storage temperature affects the VOC profile of garlic

Storage temperature significantly altered the VOC profile of garlic (PerMANOVA, \( p = 0.03, R^2 = 0.12 \)). Specifically, the VOC profile of garlic stored for 6 months at 22 °C was significantly different to garlic stored at -1.5 °C for 3 and 12 months, but not garlic stored for 6 months at -1.5 °C. Indeed, the VOC profile of garlic after 12 months at -1.5 °C was significantly distinct from all other samples (Fig. 5). Furthermore, samples stored at 22 °C exhibited a higher degree of VOC profile variability than cold stored samples at 3 and 6 months (Fig. 5).

3.8. Identifying VOCs as potential markers for storage conditions and alliinase activity

Subsets of VOCs were assessed for their potential to discriminate amongst the storage regimes.

3.8.1. Sulphur containing VOCs discriminate clearly between fresh and stored garlic

Given the importance of sulphur containing VOCs as components of the garlic profile, PerMANOVA analysis was used to assess whether this subset of VOCs alone was sufficient to discriminate amongst the samples. Significant changes in the sulphur VOC profile were found for the interaction between temperature and alliinase activity. CAP analysis showed that these VOCs discriminated completely between garlic before storage and all stored samples (Fig. 6).

The profile of garlic stored for 6 months at 22 °C was also distinct from that of garlic stored for the same time at -1.5 °C, while at 3 and 12 months the profiles were not distinct. The profile of garlic stored for 6 month at 22 °C was indeed clearly separable from the VOC profile of all other samples.

3.8.2. Use of subsets of VOCs correlating with temperature and time of storage

WCNA of the whole VOC profiles of the garlic clustered the VOCs into eight modules showing correlated patterns of change with storage time, temperature or changes in alliinase activity (Fig. 7a). One module correlated positively and significantly with storage time (blue module), one correlated negatively with storage temperature (red module) and two modules correlated with alliinase activity (black module, positively, and pink module, negatively, Fig. 7a). The blue module that correlated with storage time included 17 significantly correlating compounds: seven terpenes, three alkanes, two alcohols, two aldehydes, one alkene, one ester and one ketone; the red module correlating with temperature of storage included six significantly correlating compounds: four organosulphur, one alkane and one aromatic compound. There were four VOCs which significantly associated with alliinase activity. 4-Methylhexadecane (pink module) was positively associated with alliinase activity.
activity, having a higher concentration in samples which had high alliinase activity. Conversely, azulene, α-xylene and octanal (black module) were negatively associated with alliinase activity (Supplementary Table 4).

The compounds whose relative abundance significantly changed with storage time all peaked in relative abundance after 12 months of storage (Fig. 7b). Some compounds such as α-copaene, selinene and γ-cadinene were undetected at all time points besides 12 months (Fig. 7b). In contrast, 3-methyldecane and 3,6-dimethylloctane were only absent at 0 months, and as such their absence could indicate very fresh garlic (Fig. 7b).

The VOCs whose relative abundance associated with storage temperature were at their highest relative abundance in pre-storage samples. In the case of 3-vinyl-1,2-dithiacyclohex-5-ene, diallyl sulphide and diallyl trisulphide, this high abundance was maintained when stored at -1.5 °C, but not when stored at 22 °C (Fig. 7c). The compounds 1-methylthio-1-propene and 3-vinyl-1,2-dithiacyclohex-4-ene saw a more marked reduction in abundance when stored at -1.5 °C, and even more so when stored at 22 °C (Fig. 7c). All these compounds were less abundant when stored at 22 °C compared to when they were stored at -1.5 °C.
3.9. Assessing potential VOC subsets for their value as markers

The six VOCs varying significantly with storage temperature, as identified by WCNA, discriminated better between storage temperatures than the full VOC dataset, and temperature now explained 31.5% of the variance in the dataset (PerMANOVA). This was further explored with a CAP plot, which shows significant differences between the VOC profiles of samples stored for 6 months and 3 months at 22 °C and −1.5 °C (Fig. 8a). However, the distinct VOC profile obtained with the whole VOC dataset for garlic stored for 12 month at -1.5 °C was lost.

Similarly, the 17 VOCs associated with storage time discriminated better between storage times, and storage time now explained 36.6% of the variance in the dataset (PerMANOVA). CAP analysis shows that samples stored for 12 months had distinct VOC profiles from all other samples (Fig. 8b). However, there were no differences observed between fresh garlic or garlic stored for up to 6 months in either temperature conditions.
treatment (Fig. 8b).

Alliinase activity explained a higher proportion of the variance in the dataset than any of the other variables such as storage time, temperature and their respective interactions ($R^2$ for alliinase = 0.159, time = 0.122 and temperature = 0.149). CAP analysis was not performed due to non-categorical variables.

4. Discussion

4.1. Cold storage reduces disease

Disease incidence in garlic stored at -1.5 °C did not change from pre-storage levels, but garlic stored at 22 °C experienced a significant increase in disease incidence between 6 and 12 months. Increased disease rates were expected in storage at 22 °C, as major spoilage organisms such as Penicillium hirsutum have increased rates of spore germination, mycelial growth and infectivity to garlic at 22 °C compared to -1.5 °C (Bertolini and Tian, 1996). However, it was noteworthy that for storage durations of 6 months or less, disease rates were not significantly different at either temperature. This contrasts to the findings of Bertolini and Tian (1996), where cloves stored at 20 °C experienced a significantly higher disease rate after 4 months of storage than those stored at -2 °C. However, these results can be explained by the differences in methodologies: here, garlic was not deliberately infected with any spoilage organisms, unlike in Bertolini and Tian (1996) and so the reduced pathogen load may explain the delayed onset of disease in garlic stored at 22 °C. Furthermore, here the bulbs were stored whole, potentially conferring a degree of resistance by having intact clove and bulb sheaths, and humidity levels were lower, at 65 % ± 10 % RH as opposed to 85–90 % RH in the Bertolini and Tian (1996) study. This difference may have been paramount, as humidity over 70 % RH favours fungal spoilage organisms (Shiina et al., 2004).

4.2. Storage conditions affected alliinase activity loss

After 6 months of storage alliinase activity remained higher in 22 °C stored compared to -1.5 °C stored bulbs. Ambient storage similarly resulted in good alliinase activity maintenance in onion, where after 32 weeks, a marginal increase in activity was observed (Hanum et al., 1995). It may therefore be preferable to store garlic at ambient temperatures for biotechnological applications requiring high alliinase activity, if the storage duration does not exceed six months, although very careful monitoring for disease signs would need to be incorporated, and further work is required to validate that the disease level of garlic remains within the acceptable limits for 6 months. This also confers cost savings and reductions in carbon emissions, through negating the need to refrigerate. The increased retention of alliinase activity may also be of relevance to the food industry, since alliinase activity is essential for the synthesis of key flavour components (Jones et al., 2007).

4.3. Moisture content

Due to the destructive nature of alliinase extractions, bulb weight loss could not be measured over time, however this phenomenon is well described in the literature. Cold storage at 0 °C and 70 % RH, which was very similar to our cold storage conditions, lead to a weight loss of just 3.6–5 % over 190 d (6.3 months), whereas ambient storage over the same period caused a loss of over 20 % (Vazquez-Barrios et al., 2006). However, it was not clear from Vazquez-Barrios et al. (2006) whether this was predominantly due to a loss of water or carbon. Here, we present data that shows the moisture content of cloves remaining remarkably stable under both ambient (22 °C) and cold (-1.5 °C) storage. This expands on earlier research (Ward, 1976), which characterised moisture loss in stored Allium bulbs as a function of respiration rate, sprouting and desiccation. Additionally, the disruption of the epidermis by shoot and root growth out of the clove could increase the rate of water loss (Prange, 1998). However, here only one replicate of 3 months at 22 °C experienced any sprouting (13.7 %), with all other samples at both temperatures having no sprouted cloves (data not presented). It is therefore hypothesised that in healthy, non-sprouting garlic, weight loss through storage occurs at a rate proportional to the starting ratio of water to dry matter, which here was approximately 2:1 (64 % water to 36 % DM). The moisture content of cloves in this study was in line with that reported by Pardo et al. (2007), in which 15 cultivars were measured after harvest and had a moisture content ranging from 59.3–66.2 %, with significant cultivar effects.

4.4. Alliinase and cysteine synthase expression

Despite the effect of storage time and temperature on alliinase activity, no clear pattern was seen in the expression of the genes encoding alliinase or cysteine synthase. This suggests that the fall in alliinase activity over time during storage is post-translationally regulated. However, due to the fact that allii and other CSOs are synthesised in the leaves of garlic during growth and translocated to the bulb (Jones et al., 2007), it was perhaps expected that cysteine synthase transcripts would remain low in the bulb during storage. Cysteine synthase is a key enzyme in the synthesis of organosulphur compounds in garlic and, like alliinase,
is constitutively expressed throughout the growing cycle of garlic (Mitrova et al., 2018). Allin is synthesised both from stored S-allyl-L-cysteine during sprouting and through de novo synthesis in foliage during the growing phase (Yoshimoto et al., 2015). Our results corroborate this, with alliinase and cysteine synthase genes constitutively expressed, but not significantly upregulated at any point throughout storage.

4.5. VOCs

We report a broader range of VOCs from garlic than previous studies, with 150 compounds putatively identified after contaminant removal. Furthermore, it is not only the total number of VOCs detected which varies amongst studies, but the VOCs themselves; of 99 VOCs reported in five previous studies (Calvo-Gómez et al., 2004; Yu et al., 1989; Lee et al., 2003; Vernin et al., 1986; Molina-Calle et al., 2016), only 11 were amongst the VOCs reported here. In fact, just two compounds, diallyl sulphide and diallyl disulphide, were ubiquitous among studies and 139 compounds were unique to our study. This difference may be attributed to the fact that here, we sampled from undamaged bulbs, whereas all other studies employed some form of destructive sampling. We detected a broader range of minor VOC components, whereas the aforementioned studies predominately detected organosulfur compounds, which were likely generated from the synthesis of alliin in response to damage. Consequently, our approach did not detect compounds such as allyl methyl trisulfide (Vernin et al., 1986); thiazole, 1-propenyl methyl disulfide and methylthiocyclopentane (Calvo-Gómez et al., 2004); or 1-(1-propenylthio)propane, 5-methyl-1,2,3-thiadiazole and diallyl tetrasulfide (Lee et al., 2003) that are the product of increased levels of alliin due to mechanical damage during maceration or distillation.

The VOCs associated with storage temperature were in different chemical groups from those associated with storage time; those associated with time were mainly terpenes, ketones, alkanes and esters, whereas those associated with storage temperature were mostly organosulfur compounds. This opens avenues for the development of a VOC based quality marker which can distinguish between garlic that has been stored in different ways and for different durations. Furthermore, identifying organosulfur compounds as markers for storage temperature may suggest that temperature affects the ability of garlic to keep alliin and alliinase efficiently separated to a greater degree than storage time, as organosulfur compounds arise principally from the synthesis of alliin (Brodnitz et al., 1971). Storage time elicited the most significant changes in overall VOC profile after 12 months of storage. Instead, significant changes in metabolic status, perhaps due to dormancy break, are the likely cause for the changing VOC profile, due to the intrinsic link between a plant’s volatilome and broader metabolic status (Pichersky et al., 2006; D’udareva et al., 2004; Mansurova et al., 2018). However, further study would be needed to elucidate the pathways involved. Thus organosulfur VOCs have potential for the development of a marker system to discern fresh from stored garlic, with pre-storage garlic having a significantly different organosulfur VOC profile to all stored samples. This may be of relevance to the industry as a marker for identifying the freshest garlic, which according to our findings, will have the highest alliinase activity.

5. Conclusion

In summary we show that storing garlic leads to significant decreases in alliinase activity over time, and activity was also affected by storage temperature, showing an inverse trend to disease incidence. Whilst cold storing garlic is required to provide a year-round supply of garlic, ambient temperature storage may maintain higher alliinase activity, for up to 6 months after harvest, although further work is needed to assess disease rates during storage at different temperatures. However, alliinase activity maintenance was not regulated at the transcriptional level. Significant differences were identified in the VOC profile of garlic stored at different temperatures and organosulfur VOC bouquets showed clear differences between fresh and stored samples. Putative molecular markers were identified for storage time, temperature and alliinase activity.

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Declaration of Competing Interest

None of the authors have competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2021.111533.

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