Crop protection by volatile organic compounds from mashua: what we can learn from ancient agricultural techniques

Patricia Gonzales[1], Waltraud Kofer[1], Thais Huarancca[1], Francisco Vivanco[2], Carlos Arbizu[2] and Eric Cosio[1]

Laboratorio de Bioanalítica, Sección Química, Pontificia Universidad Católica del Perú, Lima, Peru and International Potato Center CIP, Lima, Peru
pgonzales@pucp.edu.pe

Abstract

Mashua (*Tropaeolum tuberosum*) is a perennial plant which has been grown in the Andes since prehispanic times. Despite the fact that its tubers are highly nutritious and that they can be used to cure a number of kidney ailments, mashua is limited to subsistence agriculture systems. This is probably due to its strong and pungent flavor (a result of its high levels of benzylglucosinolates) and to its reputed anti-aphrodisiac properties. Because mashua has a high resistance to bacterial, fungal, nematode and insect pests, it has been used traditionally as a companion crop to repel pests in plants that are of higher economical value, such as potato or olluco. It has been proposed that the high levels of isothiocyanates and of other volatile compounds produced by the glucosinolate pathway are at least partially responsible for this protective activity. In order to study the biochemical basis of the protection obtained from mashua plants, we analyzed volatile emission by mashua in the field and also evaluated differential volatile emission patterns (BVOC profiling) during tissue damage of 119 accessions of Peruvian mashuas grown by CIP's genebank close to Huancayo at 3700 masl, Peru. Volatiles were absorbed onto Porapak Q cartridges by active sampling using a portable air pump, were eluted from the matrix with acetonitrile and analyzed by gas chromatography on a DB-225 column with FID or MS detection. This method allowed quantification of effective field concentrations of protective volatiles and also provided a means to evaluate mashua germplasm entries based on their differential BVOC phenotype.

Introduction

Mashua (*Tropaeolum tuberosum*) constitutes the fourth most important root crop in the Andean region, after potato, oca and olluco (National Research Council, 1989). Mashua is easy to grow, produces high yields and is extremely resistant to both cold weather and pests (National Research Council, 1989, Pissard et al, 2008). Its tubers are highly nutritious; as they have a high content of carbohydrates and vitamin C (Grau et al. 2003) and it has also been reported that mashua has a higher antioxidant capacity and a higher phenolic, carotenoid and antocyanin content than other tubers (Campos et al., 2006). In addition to this, mashua has been used traditionally in the Andes to cure a variety of kidney, liver and urinary disorders and it has been recognized as an antiaphrodisiac since prehispanic times (Johns et al., 1982). Despite all the benefits it seems to offer, currently mashua is limited to subsistence agriculture systems. This is probably due to its sharp flavor, which has been attributed to its high levels of glucosinolates.

Although mashua is not exactly a commercially important tuber, it does have an important economical value: since it has a high resistance to bacterial, fungal, nematode and insect pests, it has been used traditionally as a companion crop to protect other crops such as potato or olluco from their main pests (National Research Council, 1989, Figure 1). It has been proposed that the high levels of glucosinolates in mashua are at least partially responsible for this protective activity.

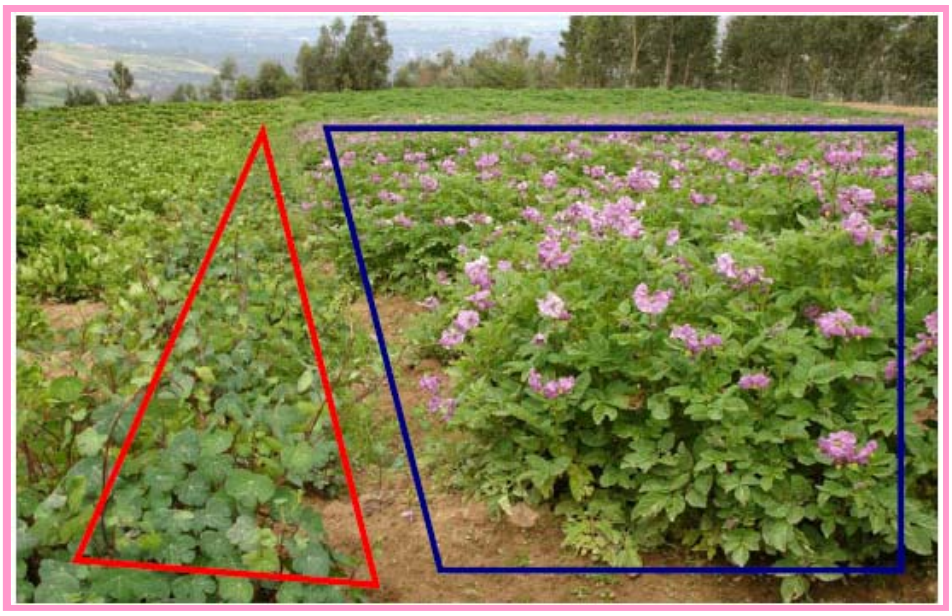


Figure 1. Potato-mashua co-cultivation system in the Peruvian Andes. Mashua (in red) is cultivated in alternated rows with potato (in blue) to protect the latter from its main pathogens

Glucosinolates are natural products that contain nitrogen and sulfur and are synthesized by plants from aminoacids (Halkier et al., 2006). Glucosinolates themselves are non-toxic, but they can be hydrolyzed to a series of compounds, such as isothiocyanates and nitriles that do have different biological effects and play an important role in plant defense mechanisms (Bennet and Wallsgrove, 1994). Glucosinolate producing plants also express thioglucosidases called myrosinases. Upon tissue damage, glucosinolates come in contact with myrosinases and are hydrolized to unstable aglycones, which rearrange to form different compounds, some of which can be toxic to insects and microorganisms (Wittstock and Halkier, 2002, Mutlib et al., 2002, Rask et al., 2000, Figure 2).

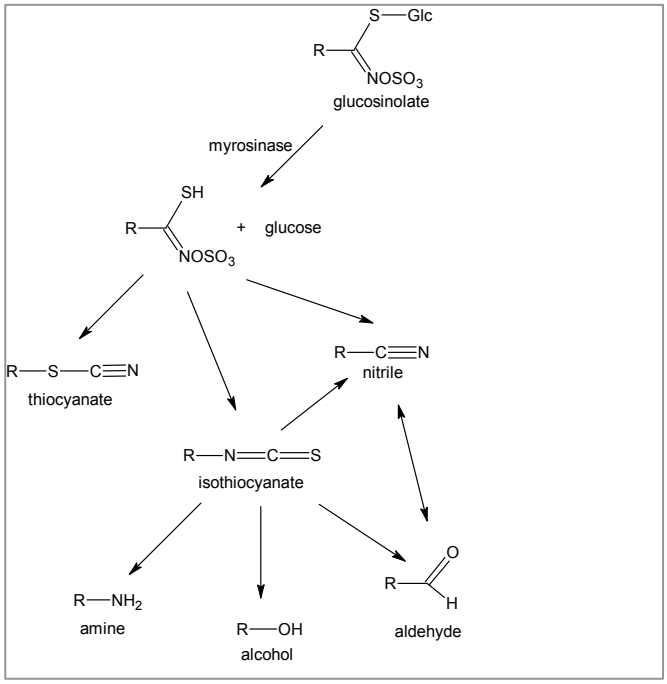


Figure 2. Products obtained from glucosinolate hydrolysis. Upon plant damage, glucosinolates are degraded to a variety of hydrolysis products. The initial step involves catalytic hydrolysis by myrosinase. R indicates a variable side chain and Glc a glucose residue

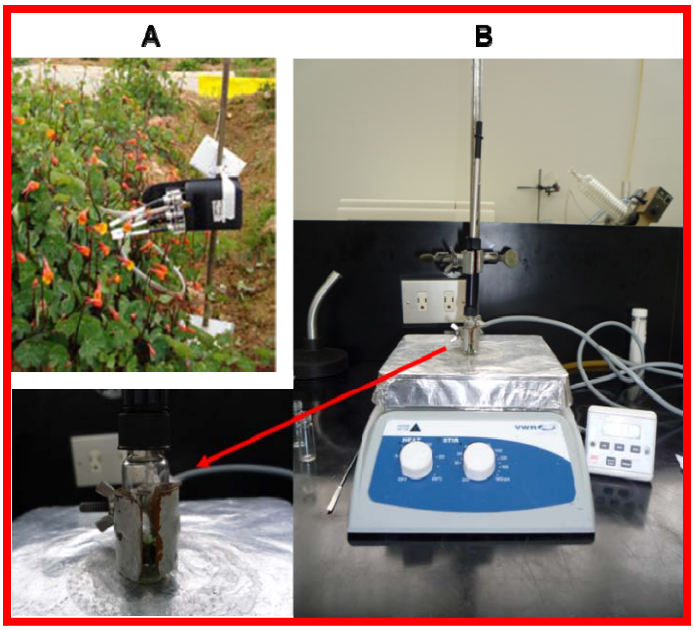
The predominant glucosinolates present in mashua are benzyl, 4-methoxybenzyl hydroxybenzyl and m-methoxybenzyl (Valer, 2001, Ortega et al., 2006) and the relative content of each of these compounds varies in the different mashua accessions that are known to date (Ortega et al., 2006). The present investigation was directed towards studying the biochemical basis of mashua´s protective activity and also towards developing a method that could allow for an evaluation of mashua germplasm entries based on their emission of volatile compounds that result from the glucosinolate pathway.

Materials and methods

Inhibition assays

Cells of *Candida albicans*, strain SC537, were provided by the European Saccharomyces Cerevisiae Archive for Functional Analysis (EUROSCARF), Frankfurt, Germany. Cells were grown to log phase in Nutrient Broth, at 30°C. Stock isothiocyanate dilutions were made in ethanol, with the following concentrations: 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28 and 2.5 µg/ml. 195 µL of dilute *C. albicans* stock were incubated for 25 hours with 5 µL of each isothiocyanate dilution in sterile plastic 96-well microtiter plates at 30°C. Following incubation, turbidity was measured at 490 nm in a ELx800 Series Universal Microplate Reader (Bio-Tek Instruments, Inc.). IC_{50} values were obtained for each of the following isothiocyanates: allyl (AITC), n-propyl (n-PITC), phenyl (PITC), 4-methoxy-phenyl, benzyl (BITC), 4-methoxy-benzyl (4MBITC) and 2-phenylethyl (2PEITC).

Field measurements of volatile emissions by active sampling



Sampling was carried out in a mashua field maintained by CIP in La Libertad, Junin, close to the city of Huancayo, at 3700 masl. Volatiles present in the field were absorbed onto glass cartridges filled with 200 mg of Porapak Q 50/80 (Supelco, Inc.) by active sampling using a portable air pump (AirLite Sampler Model 110-100, SKC, Inc.) (Figure 3A). The flow rate used was 300 mL/min and 6 L of air were captured in each case. The mashua accessions studied were DP 0224 (black mashua), M5 COL 2B, ARB 5371 and S 64. Volatiles were then eluted from the matrix with 1 mL of acetonitrile and analyzed in the PUCP laboratories, in Lima, using a Perkin Elmer Autosystem gas chromatographer equipped with a DB-225 column (30 m x 0.32 mm x 0.25 µm) and a FID detector. The temperature program used was 70°C for 1 min, followed by 70-200°C at 40°C /min and 200°C up to 6.5 min.

Figure 3. Sampling systems for mashua volatile emissions. A: Active sampling in the field using a portable air pump and Porapak Q cartridges. B: Sampling in the PUCP laboratories in Lima by headspace solid-phase microextraction (HS-SPME).

Headspace solid-phase microextraction (HS-SPME) assays

The analyses were performed in a Perkin Elmer Autosystem gas chromatographer equipped with a DB-225 column (30 m x 0.32 mm x 0.25 µm) and a FID detector. The GC temperature program used was 40°C for 4 min, followed by 40-200°C at 40°C /min and 200°C for 7 min. HS-SPME assays were performed using a manual fiber holder and a 100 µm PDMS coated fiber (Supelco Inc.). Plant material for these assays was obtained from mashua accessions DP 2124 (black mashua) and ARB 5240. These plants had been transferred to Lima and kept in pots

for 4 months. Samples were prepared by crushing approximately 0.1g of leaf or tuber material in a 4 mL screw top vial. The vial was sealed with a cap and septum and the SPME fiber was inserted through the septum and exposed for 15 minutes (Figure 3B). The compounds absorbed in the fiber were then desorbed by insertion into the GC inlet for 4 minutes at 200°C.

Results and discussion

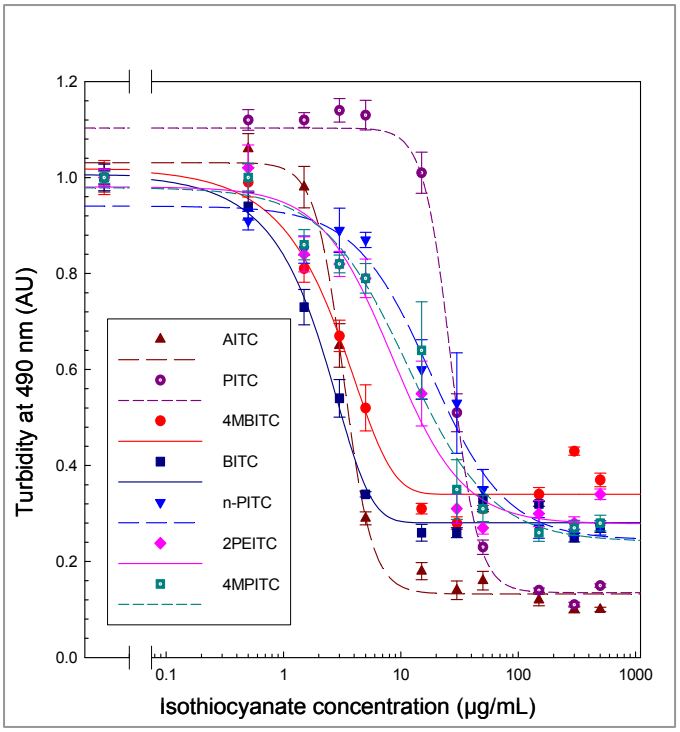


Figure 4. Antimicrobial activity of isothiocyanates. IC_{50} values were obtained using a micro titer plate based bioassay

It has long been known that isothiocyanates display antimicrobial activity. However, this issue was revisited in order to identify structural features that could be relevant for such activity. Inhibition assays performed with different isothiocyanates showed that benzyl and 4-methoxy-benzyl isothiocyanate had the lowest IC_{50} values against *C. albicans* (Figure 4).

Given the fact that benzyl and 4-methoxy-benzyl glucosinolate appear to be among the predominant glucosinolates found in mashua (Valer, 2001, Table 1), these results suggest that benzyl and 4-methoxy-benzyl isothiocyanate released by mashua in the field might be responsible for the protection provided by this plant to neighboring potato plants. A detailed assessment of the biocidal activity of these two isothiocyanates, as well as of compounds derived from isothiocyanate degradation, against the main natural potato pathogens *Phytophthora infestans, Pectobacterium carotovorum (*previously, *Erwinia carotovora)* and *Ralstonia solanacearu)* remains to be performed.

Table 1. Predominant glucosinolates present in different mashua organs of mashua accession ARB 5240. Content values are reported in µmoles per gram of dry matter (Valer, 2001). Abbreviations are as follows: 4HBGLS: 4-hydroxy-benzyl glucosinolate, MHBGLS: methoxy-hydroxy-benzyl glucosinolate, 4MBGLS: 4-methoxy-benzyl glucosinolate, BGLS: benzyl glucosinolate.

	4HBGLS	MHBGLS	4MBGLS	BGLS
Leaves	0.6	0.07	17.9	0.15
Stems	0.9	0.17	46.3	1.01
Tubers	2.4	0.45	117.1	1.16
Roots	0.3	0.63	62.9	0.43

Once we the putative defensive compounds in mashua had been identified, efforts were directed to the analysis of volatile emissions by mashua in the field. Surprisingly, the levels of isothiocyanates found in the collected samples were relatively low and the predominant emissions appeared to be benzaldehyde and benzylalcohol (Figure 5). It is possible that benzaldehyde results from further processing of benzyl isothiocyanate and benzyl

nitrile emitted by mashua (Mutlib et al., 2002). It has been observed that isothiocyanates and nitriles captured onto Porapak degrade rapidly with time (data not shown). In addition to this observation, isothiocyanates are less volatile than their corresponding aldehydes. Taking all of this into account, and considering that benzaldehyde has also been reported as being effective as a fumigant for crops (Lee et al., 2001), we propose that benzaldehyde, and not benzyl or 4-methoxy-benzyl isothiocyanates could be the main compound responsible for the protective activity of mashua, at least for aerial plant parts. We cannot discard that some amount of the benzaldehyde detected in the field could potentially originate from other plant sources such as the neighboring oca plants which, at the time of our analyses, were flowering or other nearby trees. However, the results observed from samples taken in the greenhouse confirm that mashua does emit high levels of benzaldehyde, especially when plants are in the flowering stage (Figure 5). The only plant species in the greenhouse at the time of the present investigation was mashua. More studies need to be done in which air sampling is not limited to mashua plants but is also extended to other species present in the field. We do not discard either that isothiocyanates and nitriles could still important defensive compounds in other plant organs such as tubers.

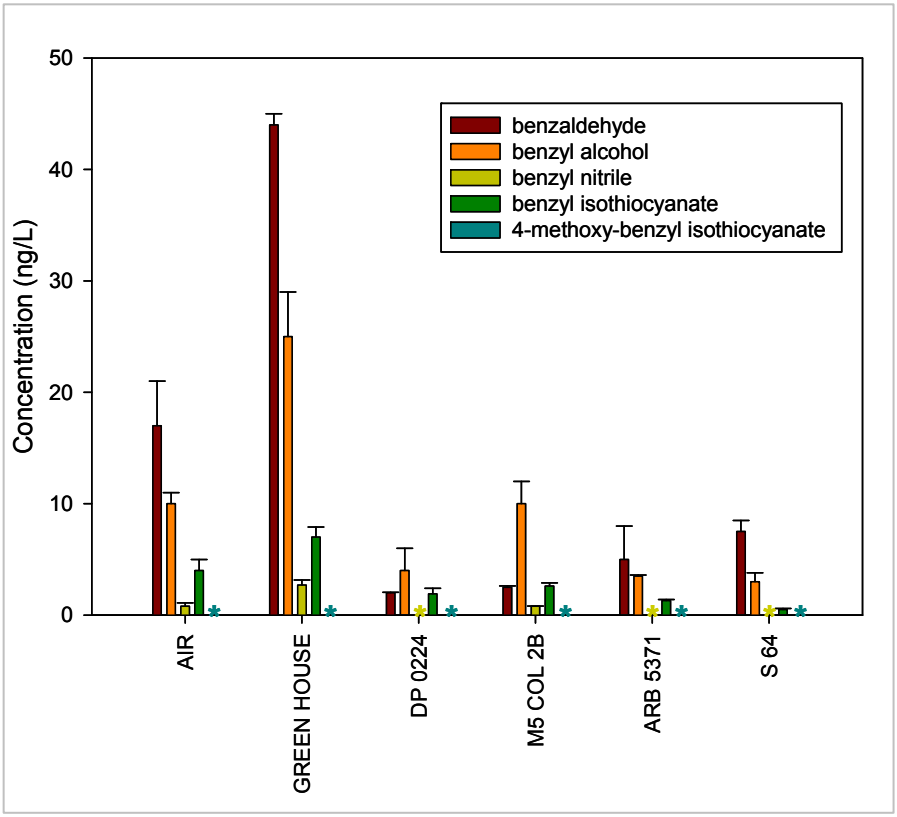


Figure 5. Volatile compounds detected in samples that were collected in the field in March, 2008. Concentration values are averages of three determinations using active sampling using a portable air pump and analysis by GC. Four different mashua accessions were studied: DP 0224 (black mashua), M5 COL 2B, ARB 5371 and S 64. Air samples from the field (AIR) and from a greenhouse were only mashua was kept were also taken Volatile compounds analyzed were: benzaldehyde (red), benzyl-alcohol (orange), benzyl-nitrile (yellow), benzyl-isothiocyanate (green) and 4-methoxy-benzyl-isothiocyanate (blue-green). The symbol (*) represents absence or very low levels of the compound, which could not be quantified.

During our studies, it could also be observed that the volatile emission patterns of different mashua accessions varied significantly (Figure 5). Therefore, active sampling in Porapak Q cartridges was also used to obtain preliminary volatile profiles of different accessions of Peruvian mashuas grown by CIP's genebank close to Huancayo (data not shown). For these analyses, volatile samples were not taken directly in the field. Instead, we used leaf material, obtained from the source plants and subjected it to mechanical damage prior to the absorption of volatiles onto the Porapak Q cartridges.

HS-SPME analyses of leaf material did not show significant levels of benzyl nitrile or benzaldehyde emission, although they did show low levels of isothiocyanate emission (Figure 6A). This could be due to the fact that the mashua plants used for the analyses had experienced a significant change in environmental conditions and they had already adapted to such conditions, as they had been kept in pots in Lima for a period of 4 months. Tuber material did show high levels of isothiocyanate and nitrile emission under the same experimental conditions (Figure 6B). However, no significant levels of benzaldehyde emission from could be observed from tubers. Since it has been proposed that benzaldehyde could be obtained upon isothiocyanate and nitrile further processing, we cannot discard that the absence of benzaldehyde in the tubers volatile profile is a result of the short emission time used in these assays (15 min). A more detailed study of the kinetics of volatile emissions from both leaf and tuber material is yet to be performed. Another possible explanation for the absence of benzaldehyde and, especially, of benzyl alcohol in the SPME fiber is that the fiber was coated with PDMS, which is a matrix of low polarity. The analyses will be repeated using a Carboxen/PDMS fiber (Supelco, Inc.), which should improve the absorption of the compounds with higher polarity emitted by mashua.

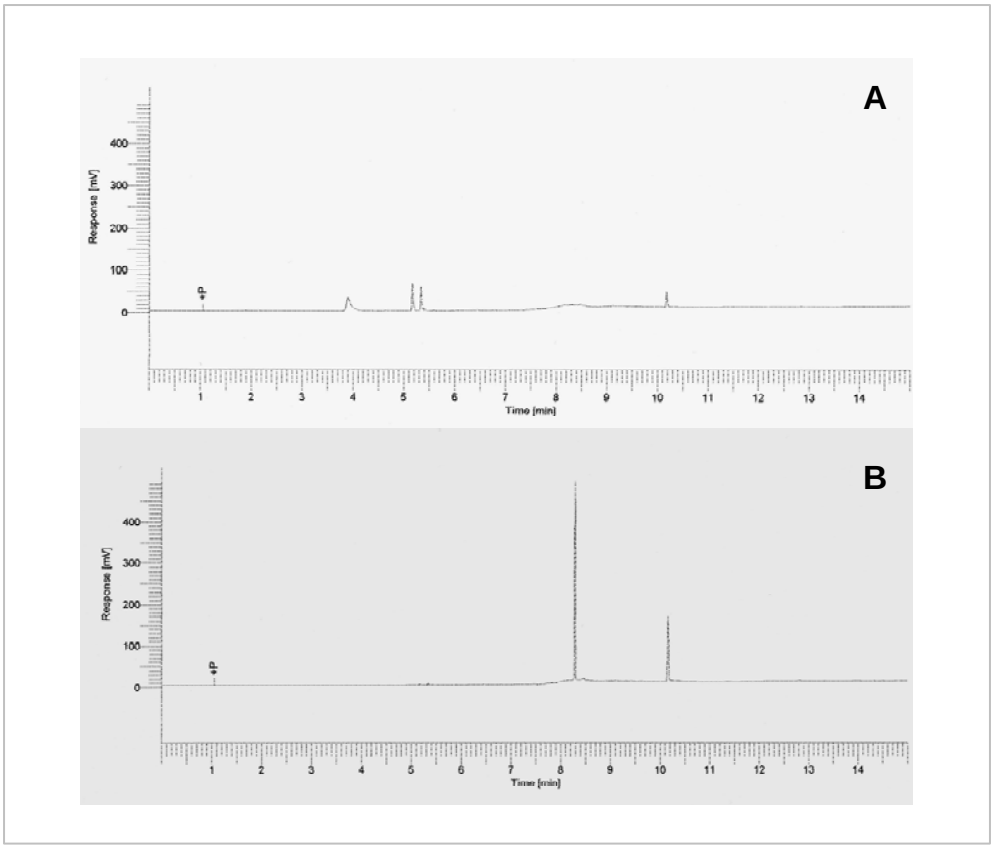


Figure 6. Volatile profiles of leaf and tuber material from mashua obtained by HS-SPME. The chromatograms show leaf (A) and tuber (B) emissions captured on a PDMS coated fiber. Compound identification was performed by comparing retention times with standards analyzed under the same chromatographic conditions.

Conclusions

Mashua has long been used in the Andes as a companion crop for its ability to repel pests from other commercially important plants such as potato and olluco. This protective activity has been traditionally assigned to the high levels of isothiocyanates and nitriles present in mashua. Our analyses of volatile compounds emitted by this plant in the field suggest that protection may also result from benzaldehyde and benzyl alcohol emissions. Benzaldehyde is also known as a defensive compound in plants and, since it has a higher vapor pressure than the aromatic isothiocyanates, it could play an important role in the protection of

mashua, especially of its aerial parts. We have also performed a preliminary evaluation of emission patterns during tissue damage of several mashua accessions grown by CIP's genebank close to Huancayo. Our results indicate that analyzing differential BVOC phenotypes in mashua is a potential tool for the evaluation of mashua germplasm entries.

References

Bennet, R.N. and Wallsgrove, R.M. (1994) Secondary metabolite in plant defence mechanisms. New Phytologyst 126: 617-633.

Campos, D.G., Chirinos, R., Arbizu, C., Roca, W., Cisneros-Zevallos, L. (2006) Antioxidant capacity and secondary metabolites in four species of Andean tuber crops: native potato (*Solanum sp.*), mashua (*Tropaeolum tuberosum* Ruiz & Pavón), oca *(Oxalis tuberosa* Molina*) and ulluco (Ullucus tuberosus* Caldas*).* Journal of the Science of Food and Agriculture 86:1481-1488.

Grau A., Ortega R., Nieto C., Hermann M. (2003) Mashua (*Tropaeolum tuberosum* Ruíz & Pav) . Promoting the conservation and use of underutilized and neglected crops. 25. Centro Internacional de la Papa, Lima, Peru / International Plant Genetic Resources Institute, Rome, Italy.

Halkier, B.A. nad Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. Annual Review of Plant Biology 57:303-333.

Johns, T., Kitts, W.D. Newsome, F. and Towers, G.H. (1982) Anti-reproductive and other medicinal effects of *Tropaeolum tuberosum*. Journal of Ethnopharmacology 5: 149-161.

Lee, B-H., Choi, W-S., Lee, S-E. and Park, B-S. (2001) Fumigant toxicity of essential oils and their constituent compounds towards the rice weevil, *Sitophilus oryzae* (L.) Crop Protection 20:310-320.

Mutlib, A., Dickerson, P., Chen, S-Y., Espina, R.J., Daniels, J.S. and Gan, L-S. (2002) Bioactivation of benzylamine to reactive intermediates in rodents: formation of glutathione, glutamate, and peptide conjugates. Chemical Research in Toxicology 15: 1190-1207.

National Research Council. Lost crops of the Incas: little known plants of the Andes with promise for worldwide cultivation, Washington, D.C.: National Academy Press, 1989, 67-71.

Ortega, O.R, Kliebenstein, D.J., Arbizu, C., Ortega, R. and Quiros, C.F. (2006) Glucosinolate survey of cultivated and feral mashua (*Tropaeolum tuberosum* Ruiz & Pavón) in the Cuzco Region of Peru. Economical Botany 60: 254-264.

Pissard, A., Arbizu, C., Guislain, M. and Bertin, P. (2008) Influence of geographical provenance on the genetic structure and diversity of the vegetatively propagated Andean tuber crop mashua (*Tropaeolum tuberosum*), highlighted by intersimple sequence repeat markers and multivariate analysis methods. International Journal of Plant Science 169: 1248-1260.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B and Meijer, J. (2000) Myrosinase: gene family evolution and herbivore defense in *Brassicaceae*. Plant Molecular Biology 42: 93-113.

Valer K. (2001) Relaciones estructura-función de la actividad antimicrobiana de isotiocianatos de Tropaeolum tuberosum (mashua) y diversos análogos funcionales sintéticos. Thesis to obtain the Licenciatura in Chemistry, Pontificia Universidad Católica del Perú. Lima, Peru.

Wittstock, U. and Halkier, B. (2002) Glucosinolate research in the *Arabidopsis* era. Trends in Plant Science 7: 263-270.