Plant biochemistry and phytochemistry

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Biochemistry and phytochemistry infiltrate the SCRI's core and externally funded programmes, complementing and enriching the skill bases in breeding, pathology and molecular biology. One of the Institute's key objectives is to harness its multidisciplinary skills in focused programmes which evolve and adapt to meet the needs of the agricultural, horticultural, and industrial communities. Programmes on environmental monitoring and protection proceed in parallel to provide an holistic approach in our science strategy. Below, we present an overview of the programmes and achievements for the past year, which embrace the skills of the Institute's phytochemists, biochemists and molecular physiologists that work alongside them.

Genotype-environment interactions and potato glycoalkaloid accumulation High-performance liquid chromatographic (HPLC) methods have been utilised to study the effects of storage temperature on the glycoalkaloid content of potato (*Solanum tuberosum* L.) tubers. The results have indicated that low temperature storage (4°C) of tubers immediately post-harvest, results not only in a cultivar-dependant increase in tuber total glycoalkaloid content but also increases the rate of glycoalkaloid accumulation upon their subsequent exposure to light. Storage at a higher temperature (10°C) prior to their movement to low temperature stores, significantly reduced glycoalkaloid accumulation in the tubers stored continuously in the dark. However, tubers stored at 10°C for a period of eight weeks followed by six weeks at 4°C, when exposed to light, accumulated significantly more glycoalkaloids than tubers of the same cultivars stored continually for 14 weeks at 10°C. The use of HPLC combined with mass spectrometry, has also been evaluated for the identification of individual glycoalkaloids. Conditions have been optimised for the production of molecular ions from both α-solanine and α-chaconine and these techniques have been applied to check the identity of glycoalkaloids detected in tubers of a number of *Solanum* species, closely related to the domesticated potato.

Novel approaches to plant lipid analysis Gas chromatography-mass spectrometry has been used to identify a number of interesting fatty acids in conifer seed oils. These include a branched-chain component, 14-methylhexadecanoic acid, which otherwise is found only in animal and microbial lipids.

The nature of the phospholipids and glycolipids in plant membranes have an impact on the physiological condition of the plant. Procedures have been developed to determine the detailed molecular species compositions of complex lipids. The polar head groups of phospholipids and glycolipids are removed by enzymatic and chemical procedures, respectively, to yield diacylglycerols. After conversion to nicotinate derivatives, the molecular species are separated according to the chain-length and degree of unsaturation of the acyl moieties by reversed-phase HPLC linked to mass spectrometry. The nicotinates have excellent mass spectrometric properties when examined both by particle-beam and atmospheric-pressure chemical ionisation (ACPI) interfaces, but the latter technique has proven more robust. The nature of the fatty acids on the glycerol backbone can be readily determined and reverse isomers (i.e. diacylglycerols with the same acyl groups but on different glycerol carbons) differentiated. Progress has also been made in analysing a variety of lipid classes (e.g. sterol esters, plant glycolipids) by normal-phase LC-MS (APCI, electrospray).

Leaf waxes and insect resistance As part of the Unit of Phytochemistry's studies of cuticular wax chemistry, techniques are being developed for determination of the spatial distribution of cuticular waxes over different parts of the plant. Differential extraction of wax from upper (adaxial) and lower (abaxial) leaf surfaces and from stems of broccoli has revealed considerable differences in the composition of wax in these regions. Waxes are usually isolated by solvent extraction, but use of a water spray to fracture and detach individual wax crystals from leaf surfaces has been investigated. This appears to extract components of the upper crystalline regions of the wax differentially, leaving the lower ones substantially intact. There are significant compositional differences between these extracts and this may be correlated with visible wax structures.

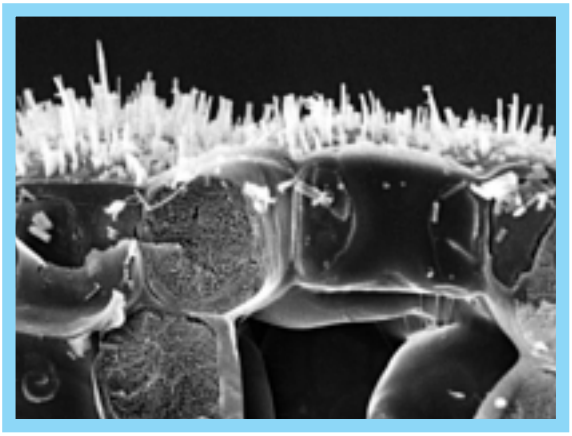

Figure 1 Leaf wax.

Work continued on the investigation of the chemistry of the leaf surface of kale, broccoli, potato, raspberry and blackcurrant. In a multiple-instrument approach, both volatile and non-volatile components have been investigated, and this necessitated the development of mass spectral data translation techniques to compare results from different instruments.

Free radicals, senescence and differentiation A component of the free radical research at SCRI is concentrating on plant senescence processes. Initial experiments have been devoted to the study of cereals, partly because of their great global importance, but also because of their architectural suitability for studies *in vivo* (which are part of the ultimate aim of this work) using the electron paramagnetic resonance (EPR) technique. Emphasis has been placed on senescence processes induced by biotic or abiotic stresses, and results show that changes in the chemical forms of iron and manganese, as well as the amounts and chemical natures of free radicals, are influenced by these stresses. This work provides a background for a more comprehensive study of the roles of free radicals and transition metal ions in different ageing processes. Over the next 3 years, this will be supplemented by an EU-funded project in which the roles of active oxygen species in plant pathogenesis will be investigated.

Also relevant to the free radical research programme is a collaboration project with researchers at the University of Abertay, Dundee. In this project, the aldehydic products of lipid peroxidation, malondialdehyde and 4-hydroxynonenal, have been profiled in callus cultures of *Daucus carota*. This is the first occasion that the latter has been detected in plant tissues. Clonal lines differing in embryogenic potential have shown different contents of the two compounds, and indeed different ratios. Both the absolute contents and the ratio have been shown to be good indicators of embryogenesis.

Stable isotopes technologies and applications Continuous flow analysis of sulphur stable isotopes is now a routine analytical procedure within the stable isotope facility in the Chemistry Department. The methods described for sulphur-rich minerals (SCRI Ann. Rep. 1994, 97-100) have been extended to include a range of biological samples with sulphur contents between 0.1 and 1%. This is achieved by ensuring rapid and complete sample conversion by the addition of vanadium pentoxide to the samples, and allowing only pure sulphur dioxide to enter the mass spectrometer. Samples analysed successfully include invertebrates and plants from studies of both terrestrial and marine food webs. The timing of sulphur uptake and reallocation in wheat plants throughout the growing season has been studied using this analytical approach and sulphur sources with naturally different ^{34}S content. Experiments covering the growing season are best done with stable isotopes due to the short half-life and radiation hazard of ^{35}S. Rapid, automated stable isotope analysis makes such studies a practical proposition. An accompanying, fuller article outlines developments in the interpretation for $\delta^{15}N$ in plants.

Plant fibres, plant cell walls and crops for industrial use Within the Unit of Plant Biochemistry, the activity of enzymes capable of oxidising and polymerising monolignols has been shown to be present in cell wall-associated proteins from the developing xylem of a range of taxonomically-diverse trees. This ubiquity of monolignol-specific oxidases indicates that they are required for lignin formation. Their rôle is not fully understood but the initial products of monolignol oxidation formed by these oxidases are not significantly different from those obtained by oxidation with peroxidase/hydrogen peroxide. This suggests that they do not make lignin with different sub-unit linkages. The coniferyl alcohol oxidase from Sitka spruce has been purified and amino-terminal sequence obtained. Electron paramagnetic resonance spectra of these oxidase-enriched extracts contain signals indicative of a free-radical and type II copper atoms. The free radical signal appears to be involved in the catalytic mechanism of the oxidase, as its intensity is diminished by addition of coniferyl alcohol and is re-established by reaction with oxygen. The free radical intermediate has properties that are different from those of other known free radical intermediates of plant enzymes. The involvement of a bound free radical intermediate may explain the usually broad substrate specificity of this oxidase.

Work has been initiated on the identification of xylotransferases involved in the biosynthesis of the major secondary cell wall polysaccharide, xylan, and their purification using protocols previously established at SCRI. Developing flax xylem has been identified as a good source of this enzyme, as it is present at much higher specific activity with lower contaminating glycosyl transferase activities than previously used model systems such as pea.

Many transgenic lines of tobacco with genetically modified lignins have been analysed to assess the effect of modification of gene expression on cell wall composition and structure. Transgenic plants studied have modified activities of cinnamyl Co-A reductase and feruloyl-5 hydroxylase (in addition to previously reported work with cinnamyl alcohol dehydrogenase). Modified expression of these enzymes has a distinct effect on the constitutive lignin formed. For example, levels of lignin cross-linking have been reduced which will facilitate easier pulping.

The studies on Reed Canary grass as a potential source of pulp for paper and biofuel, continue. The varieties

Figure 2 Reed Canary grass.

grown have all become well established, giving potential yields well in line with those of other EU partners. The analyses of the quality parameters being determined at SCRI for material for the 1996/97 harvest have been completed but the interpretation and prediction of the data has still to be carried out.

The Crops for Industrial Use project (FIBSTORE), designed to determine the effects of storage regimes on baled straws, has been completed. Straw from different regions of a large stack, with a range of moisture contents, has been analysed chemically, spectroscopically and for microbial spoilage. In summation, it has revealed that storage outdoors causes bales on the outside of stacks to rot. Biodegradation is due to the action of fungi and their removal of hemicellulose (also causing some damage to cellulose microfibres). Treatment with biocide affords some protection initially, but this effect is lost with time.

The use of novel bleaching agents for use in the pulp and paper industry has continued with the work on oxone now completed. This successfully bleached/delignified all of the sources of annual fibres examined with the exception of oilseed rape straw. Promising results have been obtained using the metal ion chelator tetraacetylethylenediamine (TAED) in combination with hydrogen peroxide.

Starch: genetic variability and genetic engineering Continuing the theme of crops for industrial use, a SOAEFD Flexibly Funded project has been aimed at identifying the processing potential of starches from barley, wheat, oat and potato varieties/cultivars grown under Scottish conditions. This has required detailed comparative assessments of starch structure and rheology. Part of the programme has investigated barley starches with different genetic backgrounds, especially waxy and high-amylose types. With potatoes, the variation in composition and properties has been shown to be high. Variation between variety/cultivar appears to be greater than between growing sites. For example, a two-fold difference in phosphate ester content between varieties/cultivars is possible while even larger differences in viscosity parameters occur.

Complementing the above, a detailed analysis of transgenic potatoes expressing, ectopically, a range of genes with the potential for modifying starch structure, is well underway. One gene previously isolated as an α-glucosidase by yeast complementation, has been identified as a plant equivalent of glucosidase II, an enzyme essential for glycoprotein processing in eukaryotes. This is the first time an enzyme of this class has been identified in higher plants. As in mammals, the enzyme is enriched in the microsomal fraction of non-transgenic plants. Transgenic plants with the gene down-regulated show an extremely stunted phenotype. More recently, an additional α-glucosidase gene has been cloned which is more similar to those believed to be involved in primary carbohydrate metabolism.

Also relevant to the future manipulation of starch synthesis and structure is the elucidation of the pathway of (floridian) starch biosynthesis in the red alga *Gracilaria tenuistipitata*. Unlike in higher plants and green algae, the elongation of polymeric α-1,4 glucan chains occurs via a unique glucosyltransferase specific for UDPglucose. This novel UDPglucose-starch synthase has been partially purified and characterised and a putative N-terminal amino acid sequence obtained. A novel HPLC-based method for the assay of starch synthase has also been developed during the course of this work. The method is highly sensitive and does not require the use of radiolabelled substrates.

Developmental processes: biochemical, molecular and NMR imaging approaches Other aspects of carbohydrate research are relevant to developmental processes such as dormancy break and sprout growth in potato tubers (EU-funded programme). It has been established that initial bud growth is sustained by the mobilisation of soluble compounds from the tuber. This phase is followed by the onset of active starch degradation in the tuber which corresponds with the expression of a new β-amylase isoform and a large increase of soluble sugars. Sprout removal induces very rapid changes (within hours) in carbohydrate metabolism in the tuber, causing an induction of gly-

Figure 3 NMR of a blackcurrant.

colytic metabolism and starch biosynthesis. These changes occur prior to any quantifiable change in tuber sugar content and indicate the existence of a highly co-ordinated sink-source relationship between tuber and sprouts.

Within the Unit of Plant Biochemistry, several soft fruit biotechnology programmes have been initiated this year and funding has been secured from a wide-range of sources including growers associations, industry, SOAEFD and the EU. Genes involved in fruit ripening have been isolated from strawberry and raspberry and already novel targets for the transgenic phases of these programmes have been identified. Efforts to understand the changes in carbohydrate biochemistry that occur during strawberry fruit ripening are also underway and a major programme designed to improve ascorbate levels in blackcurrant fruit has been established. An overview of progress on the chemistry, biochemistry and molecular biology of raspberry fruit ripening is provided in an accompanying review article.

An NMR-imaging, developmental study of blackcurrant fruits from flower to mature fruit has been completed. NMR images were corroborated by low temperature SEM and resin section light microscopy and included a 3-D time course of a fruit still attached to the plant. Gradient echo images revealed the 3-dimensional structure of gelatinous sheaths around the seeds and their vascular traces in over-ripe fruits. The seemingly paradoxical change with maturity in image contrast of the vascular bundles, was ascribed to changes in cell sizes and intercellular gas spaces around the vascular bundles which affected the magnetic susceptibility homogeneity.

In collaboration with the Germplasm Conservation Department at RBG Kew, the 3-D distribution of mobile protons and 2-D water and lipid distributions in the West African seed *Vitellaria paradoxa* have been monitored during dehydration and subsequent rehydration. The mobile water content never regains its initial level and its distribution is more amorphous. The gross water and lipid NMR signal intensities appear to correlate with differential scanning calorimetry measurements of water mobility.

NMR imaging has also been used to investigate water imbibition by different cultivars of malting and malted barley. This work was carried out in collaboration with the International Centre for Brewing and Distilling at Heriot-Watt University and preliminary studies have indicated that there are differences in the rate of water uptake and the distribution of water in the endosperm between samples of 'good' and 'poor' malting barley and malt grains. Also using seeds, a comparison has been undertaken of germinating *Vicia faba* seed at two different magnetic field strengths (0.47T at Wageningen NMR Centre and 7.1T at SCRI). At high field, the best contrast, which revealed internal structure, was obtained using an unweighted spin echo sequence; little discrimination was obtained at low field under the same conditions. Good visualisation of the same features was obtained from low field longitudinal relaxation rate ($1/T_1$) images, although not as dramatic as in the high field images. The greater contrast in the spin echo images at high field is probably due to decrease in signal from the endosperm as a consequence of chemical exchange of protons between water and starch. This signal attenuation will be much less prominent at low fields, giving rise to a higher overall signal and less discrimination between the tissue types.