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Phytochemical resistance-traits in crops against pests and diseases

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ABSTRACT

The phytochemical-resistance trait comprises of is one or more compounds produced by the crop for resistance against pests and diseases. Breeding for phytochemical resistance-traits may offer more stable crop resistance and less dependance on pesicides.. Nevertheless, incorporation of phytochemical resistance-traits in crop improvement faces several problems viz., monitoring during breeding process and their biological impact on agroecosystems. This Paper describes several methods for easy monitoring of phytochemical resistance-traits in crop species, including the rapid and often cheap assays to measure contents of glucosinolates, cyanogenic glycosides, phenylpropanoids, alkylresorcinols, resveratrol, salicylic acid and activity of phenylalanine ammonia-lyase. These methods should be used complementary to visual traits of resistance such as ratings of disease, or insect feeding. They can provide a better understanding of the underlying mechanisms involved in crop defense to pests and diseases.

Keywords: Diseases, pests, phytochemical resistance-trait, secondary metabolism

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1. INTRODUCTION

Although modern breeding of crops has ensured high yields and better quality of harvestable organs, but decreased the restance to pests and diseases (13,34). However, the intraspecific variability of crops stored in their wild ancestors, inbred lines and landraces. Therefore seed banks have been established worldwide to preserve these valuable resources, to incorporate them in breeding programs for resistance to pests and diseases (32). Screening for resistance traits is a laborious and long term process that requires the performance of recurrent bioassays and field trials challenging the plants with a certain pest or pathogen. Selection of desired resistance phenotype often is based on few visual handful traits such as disease severity or insect feeding (15). In this situation, genetic variation of crop species has been exploited mainly as source of monogenic resistance traits and underexploited for quantitative polygenic resistance ones. The formers are phenotypically visualized as strong qualitative differences between susceptible and resistance crop genotypes. The latters are the mostly observed and consists of quantitative and gradual phenotypical differences in susceptibility among crop genotypes (19). In later, handful traits do not indicate the physical and/or chemical factors involved in resistance. The identification of these factors is of increasing interest because they can help to understand the genetic mechanisms controlling disease or pest resistance, and how to use them for crop breeding (28).

2. PHYTOCHEMICAL RESISTANCE-TRAITS

High levels of secondary metabolites in plant organs/tissues have been implicated as crop traits in the resistance against pests and diseases (11,14,30). Selection for high content of some secondary metabolites may offer the advantage of a more stable crop resistance or less dependance on pesicides. Nevertheless, the use of these phytochemical resistance-traits in plant breeding faces following problems:

(i) Monitoring of a phytochemical trait associated to crop resistance requires to clearly know when and where the metabolites should be sampled. For example, the phytochemicals should be extracted in the plant stage and organ tissue susceptible to the pest or disease. The mechanisms of chemical defence strategy should be also considered: some metabolites can be constitutively expressed (i.e. phytoanticipins) or are synthesized after pathogen or pest attack (i.e. phytoalexins). Differences between both strategies sometimes is not so evident because several secondary metabolites has a phytoanticipin role but also increase their levels after pathogen or pest attack (8). Also, a molecule with phytoanticipin role in a crop species sometimes has a phytoalexin role in another one. Specific cell types involved in metabolite synthesis (i.e. sorgoleone in sorghum hair roots, essential oils often in surface trichomes, etc), cell compartments (i.e. vacuoles, plastids, etc) or sites of accumulation (i.e. extracellular surface waxes, located zones sorrounding pest or pathogen attack) should be sampled. Sometimes the secondary metabolites are released outside the plant in the form of seed, root exhudates and leaf volatiles or leachates (1).

(ii) Most phytochemical traits are connected to crop resistance by a correlation relationship (i.e.. a higher level of a metabolite sometimes means less susceptibility), but the undelying mechanism of chemical defense *in vivo* is often unknown. Deep ecological studies indicating the pleiotropic impact of high or low phytochemical traits on the multiple crop-organisms interactions occurring in the agroecosystem often are not available. In this situation, a good source of the potential impact of phytochemical traits may come from research on the wild living ancestors of the crop plants.

(iii) In phytoanticipins, sometimes the resistance trait is for high level of a breakdown product or high activity of an hydrolitic enzyme instead of high phytoanticipin content. For example, nitrile and isothiocyanate are the breakdown products of 4-methylsulfinylbutyl-glucosinolate. Originally it was though that breeding for high levels of 4-methylsulfinylbutyl-glucosinolate (4-MSBG) would provide resistance to generalist insects. Nitrile shoed to be an attractant and isothiocyanate a deterrent of generalist herbivores. Genetic variation in crucifers exist on how the glucosinolate is broken down, with predominance of either nitrile nor isothiocyanate release. Hence, the resistance effect is associate to high nitrile content (or high activity of the hydrolitic enzyme responsible of nitrile release) instead of high 4-MSBG (16).

(iv) Chemical resistance of the crop plant sometimes is better depicted by the contents of a mix of compounds with different or similar chemical nature than by the content of a compound alone. Hence, chemical analysis for plant breeding should take account global contents of secondary metabolites instead of individual ones (28).

(v) Selection for high levels of some defense metabolites can contribute to undesirable traits (i. e. bitter taste, increased hardness, toxicity for human and cattle) which reduce human acceptance of harvestable organs. In such cases, crop has been selected for low contents of defense metabolites (i. e. low levels of cyanogenic glycosides in sorghum, glucosinolates in rapeseed and saponins in alfalfa).

(vi) Quantification of a high or low-metabolite trait should be performed by rapid, unexpensive and easy-to-use methods sensitive enough to work with small amounts of plant material.

This Paper provides experiments used to asses the phytochemical resistance-traits. They are tools complementary to visual field traits applied for screening of crop resistance to pests and diseases.

3. EXPERIMENTAL METHODS

3.1. Glucosinolates

The glucosinolates are sulfur containing compounds. After hydrolysis, they provide the pungent aroma and bitter taste of cruciferous species as broccoli, radish and cauliflower (4). In intact tissues of cruciferous plants, glucosinolates and the thioglucosidase enzyme myrosinase accumulate in different cell compartments. The enzyme is in contact with glucosinolate only after tissue damage, where it cleaves off the thio-glucose from the glucosinolate molecule (6). This hydrolysis releases a short life aglycone readily degraded to nitrile, isothiocyanate and thiocyanate (Figure 1). Glucosinolates ae deterrents for vertebrate herbivores and generalist insects that do not

specialize on cruciferous plants (17,18). Some specialized phytophagous insects are able to detoxify these molecules. Breeding of cruciferous crops for either low or high glucosinolate levels might not alter total herbivory resistance but instead modify the insect herbivore populations affecting the crop itself. Cruciferous species such as rapeseed have been improved for low glucosinolate content to allow their use for livestock feeding (14).

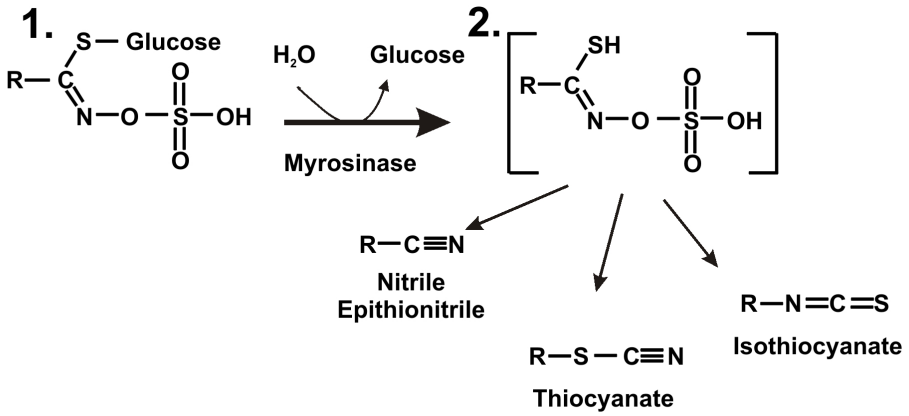


Figure 1. Degradation of glucosinolates after crushing of plant tissues: (1) General basic molecular structure of a glucosinolate. (2) Short live glucosinolate aglycone released by myrosinase, is degraded to nitrile, thiocyanate and isothiocyanate.

Experiment 1. Glucosinolate levels in cruciferous crops

Principle

Several simple methods allow the screening for glucosinolates. Some of these methods measure the equimolar amounts of glucose released from glucosinolates by endogenous myrosinase. Glucose is quantified using a blood glucose strip paper. These strips contain the enzymes glucose oxidase (EC 1.1.3.4)/peroxidase (EC 11.1.1.7) together with o-toluidine and a yellow dye (FDC yellow # 5). The reactions involved are summaryzed in Figure 2. In presence of glucose, the yellow strips turn to green tones.

Materials

Seeds from rapeseed accessions with high and low glucosinolate contents, commercially available blood glucose strips, 0.1 ml glass pipette or a 0.1 ml micropipette, mortar and pestle.

Procedure

(i) Powder five rape seeds in a mortar with a pestle. Then, add 100 µl distilled water. Ensure a good homogeinization.
(ii) Add the homogenate to the strip and left for 3 minutes.
(iii) Compare the developed colour to the colour scale provided by the strip manufacturer. The strips can distinguished glucose contents between 0 and >2%.

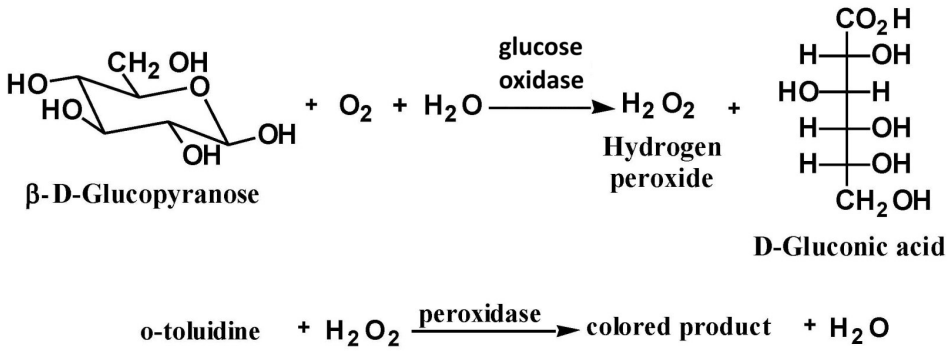


Figure 2. Reactions involved in the glucose oxidase/peroxidase kit containing o-toluidine and FDC yellow dye.

(iv) Glucosinolate content (%) is the same as the glucose content (%) determined with the strip (see Observation).

Observations

Endogenous glucose levels in rape seeds should be determined before estimating the glucosinolate content. To do it, inactivate myrosinase by placing rapeseeds at 110 °C for 12 h. Then, follow the procedure indicated above for un-heated seeds. In this case, glucosinolate levels corrected (GlC_c) for endogenous glucose content (G) should be calculated as under:

$$GlC_c\ (\%) = GlC - G$$

Where, GlC: Glucose released after glucosinolate degradation determined in unheated seeds.

3.2. Cyanogenic glycosides

Cyanogenic glycosides are cyanide containing compounds which occur in about 2000 plant species, including several forages [birdsfoot trefoil and white clover], edible plants [cassava, sorghum, almond] and several stone fruit species such as [cherries, apples, almonds, peaches and apricots (12)]. When the glycoside is hydrolysed, the aglycone released is unstable and cyanide is released in what is a defence response against several microorganisms, insects and vertebrate herbivores (Fig 3). Poisoning of cattle grazing on cyanogenic forage species and humans after consumption of poorly prepared cassava has been reported (21). Cyanide is highly toxic, because it binds to the haem group of the cytochrome oxidase located in the mitocondria, the final step in oxidative respiration. Plant producers avoid autotoxicity by the spatial separation of the stable cyanogenic glycoside and the enzyme β-glucosidase responsible of its degradation. Glycoside and the glucosidase are brought together when plant is crushed or chewed (20). Breeding for low level of cyanogenic glycosides can determine their contents by direct or indirect methods. The formers imply instrumental analysis (i. e. Liquid chromatography coupled to mass spectrometry) and the latters depends on the degradation of glycoside and then the assessment of cyanide or glucose released.

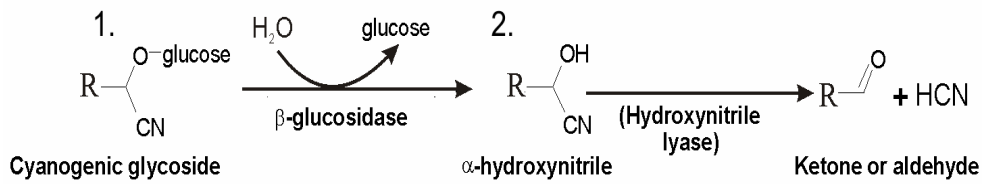


Figure 3. Release of cyanide from crushed plant tissues: (1) General basic molecular structure of a cyanogenic glycoside. (2) A β-glucosidase catalyses the release of a short live α-hydroxynitrile intermédiate which is degraded to ketone or aldehyde and cyanide by a hydroxynitrile lyase.

Experiment 2. Guignard sodium picrate test

Plant material is crushed and placed into a closed container which has a paper strip embedded in sodium picrate. The endogenous b-glucosidases hydrolyse the cyanogenic glycosides and the volatile cyanide is released. Red sodium isopurpurate is formed in the strip by reaction of the cyanide evolved with the sodium picrate (21).

Materials and equipment

Fresh plant tissue (i.e. birdsfoot trefoil, apple seeds or almonds). Mortar and pestle, sand or liquid nitrogen (to facilitate grinding); Flat bottomed glass specimen tubes (50 × 10 mm), with corks. Strips of filter paper (Whatman # 1; 38 × 8 mm). Forceps. Filter paper (approx. 120 mm × 120 mm, or 90-mm disks). Petri dish (6 cm diameter).

Reagents

Sodium picrate.

Procedure

Preparation of Guignard test papers

(i) Prepare sodium picrate (26 mM): Dissolve 5.7 g of picrid acid in a liter of distilled water. Add 50 g of Na_2CO_3 (bubbling indicate the neutralization of picric acid is in progress). Filter and store in a dark bottle with a screw cap.
(ii) Place 15 ml sodium picrate solution in a Petri dish. Using the forcep, immerse the paper strip in the solution.
(iii) Dip the strips and use for the test

Using the test papers

(i) Crush or finely grind samples in a mortar and pestle using 5 ml liquid Nitrogen or some sand as an abrasive (see Observation 1).
(ii) Quickly place the tissue in the base of a tube, moisten with water or a buffer (see Observation 2).
(iii) Fold one end of a test paper over the top edge of the vial and hold in place by screwing on the lid. It is important to do this quickly because once the plant tissue is crushed, the cyanogenic glycosides will start to break down and release cyanide. Take care not to let the papers get wet or to actually touch the tissue.
(iv) Papers turn purple in the presence of cyanide. Record the colour of the test paper after 0.5 h, 2 h and 24 h on a scale of 1 to 5 (see Observations 3, 4 and 5).

Observations

1) **Cyanide is volatile at 27 °C so generally these steps are performed quickly and on ice**.
2) The water used to moisten the plant tissue can be replaced with buffer (0.1 M citrate or phosphate buffer, pH 5.5-6.8). Non-specific β-glucosidases can be added to the buffer to test for presence of cyanogenic glycoside in the absence of endogenous degradative glucosidases in the tissue being tested (see Experiments 3-5).
3) The speed and intensity of the colour change is an indication of the concentration of cyanogenic glycosides.
4) Readings should not be extended beyond 24 h because of the risk of false positive tests through the action of cyanogenic bacteria. For longer periods, an antibiotic should be added.
5) Level of cyanogenesis can be measured using a colour chart with variations of yellow, orange, and red colour, based on concentrations of KCN. Improper handling of KCN can be lethal because it releases cyanide at room temperature.

Precautions

Dry picric acid is explosive, be careful to mantain it wet. Always wear gloves and forceps when handling the test papers. Before handling liquid Nitrogen or KCN, ensure you are familiar with safety information and recommended personal protective equipment advised by your commercial provider.

3.3. Phenylpropanoids

Cereals and grasses have high concentrations of ferulic and *p*-coumaric acids ester-linked to cell wall polysaccharides. Diferulate isomers (DFAs) are formed during cell wall deposition and lignification by peroxidase-mediate coupling of the ester-linked ferulate monomers. They cross-link cell wall polysaccharides. Crosslinking increases cell wall hardness and may act as a structural barrier restricting pathogen infection and pest feeding in intact stalk, leaf or kernel pericarp tissues of some cereals (31). Negative correlations have been found between content of DFAs in tissues of cereal crops and disease severity or insect damage. This situation suggests that DFAs are an important component in cereal resistance to pests and diseases. For this reason, high DFAs trait is of increasing interest in cereal breeding. Cell wall DFAs also showed *in vitro* inhibitory activity on mycotoxin biosynthesis, which was as effective as ferulic acid (5). They might exert this inhibitory activity after release by fungal esterases and other extracellular enzymes during fungal infection.

Experiment 3. Pericarp phenylpropanoids from cereal kernels

Principle

Phenylpropanoids from pericarp kernels of maize are released from cell walls by alkalin hydrolysis, extracted with an organic solvent and quantified by HPLC coupled to a diode-array detector (30).

Materials and equipment

Healthy kernels from maize genotypes previously characterized for disease resistance in the field (i. e. against Fusarium ear rot). HPLC system coupled to a diode array detector and equipped with a 20 µl loop and a C18 column (25 cm x 4.7 mm, 5 µm particle size). Scalpel. Lyophilizer. Rotary evaporator. Wiley mill. Disponsable celullose filter membranes (0.45 µm pore size). Glass pipettes and micropipettes.

Reagents

Standards of *trans*-ferulic and *p*-coumaric acids (see Observation). HPLC grade water, acetonitrile and formic acid. Solutions of 2 N NaOH and 12N HCl.

Procedure

(i) Dry and shell maize ears with no visibly diseased kernels. Prepare a composite samples from equal weight subsamples of kernels from each ear. Store composite samples at 4°C until processing.

(ii) Freeze dry the composite kernel samples and then soak them for 4 h at 4°C. Separate the pericarp layer from the endosperm of the hydrated kernels with a scalpel. Then, powder the excised pericarp in a wiley mill and freeze dry.

(iii) Digest 5 g of pericarp powder with 60 ml of 2N NaOH for 3 h in darkness, with mixing at half-hour intervals. Then, adjust to pH 2.0 with 16 ml of concentrated 12N HCl, vigorously mix and extract twice with 100 ml of ethyl acetate.

(iv) Combine the organic fractions and dry in a rotary evaporator. Dissolve the dry sample in 3 ml of high-performance liquid chromatography (HPLC) grade methanol and stored at 18°C until HPLC analysis.

(v) Filter the methanolic extracts through a cellulose filter with 0.4-µm pore size. Inject 20 µl in an HPLC coupled to a Diode Array Detector, using a C18 column at a flow of 1 ml/min. Use a binary gradient as mobile phase with solvent A (2% formic acid in water) and solvent B (2% formic acid in acetonitrile) in the ratio of 0% B for 0.38 min, increasing to 20% B in 14.62 min, increasing to 75% B in 15 min, and mantaining at 75% B for 5 min.

(vi) Phenylpropanoids are identified based on standards and UV-Vis spectra (Figure 4) and quantified using the external standard method (30)

Observations

Standards for diferulates are not commercially available. Small samples can be provided by the Laboratory of Biology of Bioactive Agents and Phytopathogens (LABIFITO – Tucumán, Argentina).

3.4. Alkylresorcinols

5-n-Alkylresorcinols (5-ARs) are phenolic lipids. In higher plants, the main known sources of 5ARs are kernels of winter cereals (i.e. barley, wheat, rye and triticale) and fruits of *Anacardium occidentale* and *Ginkgo biloba*. Homologues of 5-ARs are accumulated in the outer layers of winter cereal kernels (Fig. 5). For this reason, bran and whole grain products of wheat and rye contain ARs, while they are absent or found in very small concentrations in endosperm derived products (26). High 5-ARs content have been often indicated as a resistance trait of winter cereals to fungal pathogens and can be easily assessed by colorimetric methods. The most widely used are based on the diazonium salts

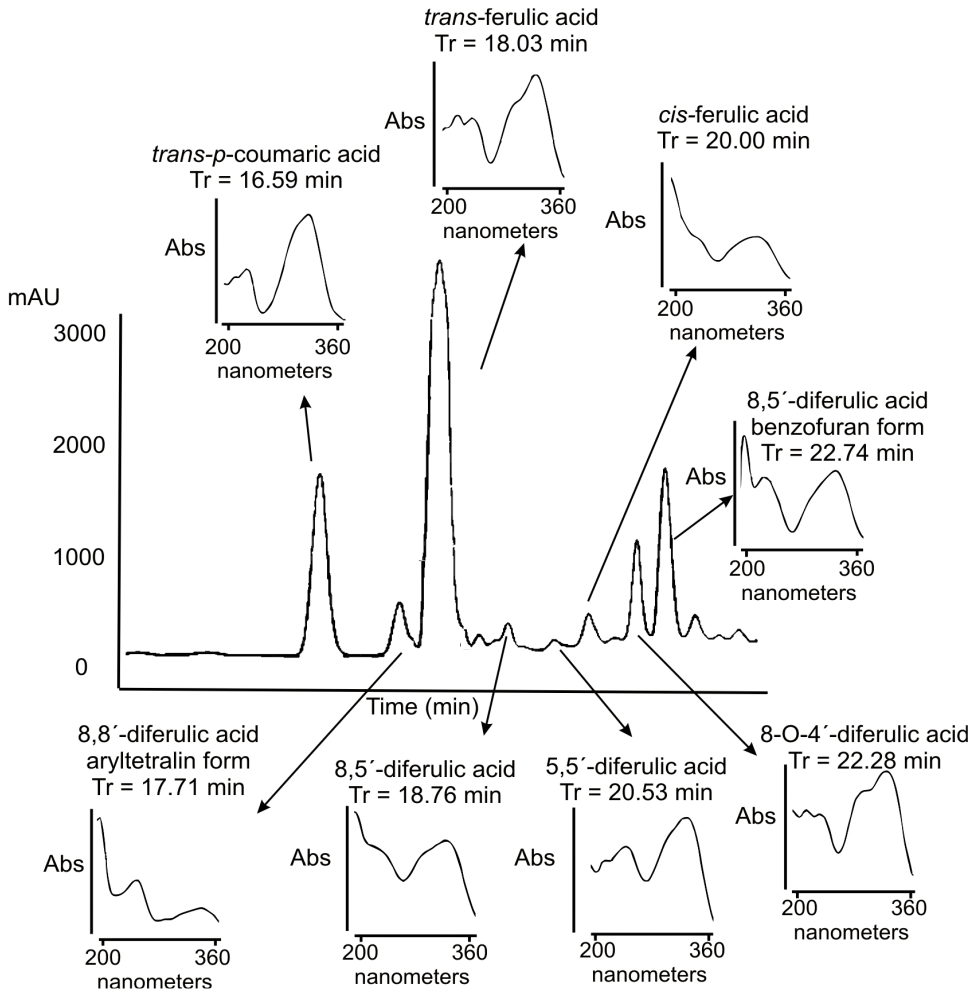


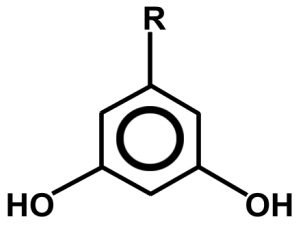
Figure 4. HPLC chromatogram of a typical pericarp sample and UV-Vis spectra of main phenylpropanoids detected. Tr = retention times.

Fast Blue B (10) and, more recently, on Fast Blue RR (27). Both diazonium salts showed great specificity for 5-ARs. They can quantify 5-ARs in good agreement with gas chromatographic-mass spectrometry methods.

Experiment 4. Fast Blue RR method for alkylresorcinols in kernels of winter cereals

Principle

The diazonium salt Fast Blue RR in basic medium copulate with alkylresorcinol molecules having both free hydroxyl groups attached to the aromatic ring in the meta



R	name
$C_{15}H_{31}$	5-*n*-pentadecylresorcinol, cardol (C15:0)
$C_{17}H_{35}$	5-*n*-heptadecylresorcinol (C17:0)
$C_{19}H_{39}$	5-*n*-nonadecylresorcinol (C19:0)
$C_{21}H_{43}$	5-*n*-heneicosylresorcinol (C21:0)
$C_{23}H_{47}$	5-*n*-tricosylresorcinol (C23:0)
$C_{25}H_{51}$	5-*n*-pentacosylresorcinol (C25:0)

Figure 5. Chemical structure of main alkylresorcinols commonly found in cereal grains.

position. This reaction yields reddish-violet products with maximum absorbace at 490 nm (27). Co-extracted compounds that may interfere with the colorimetric method can be eliminated by appropriate selection of extraction solvents and by flash microfiltration on silica gel. The following procedure is appropiate for quantification of 5-ARs in cereal kernels.

Materials and Equipment

Kernels from winter cereals (i.e. wheat, barley and rye); Glass pipettes, micropipettes. Assay tubes. Filter paper. Rotary evaporator.

Reagents

Stock solution of 0.05% Fast Blue RR reagent: Dissolve 50 mg of Fast Blue RR (½$ZnCl_2$) into 100 ml of methanol. **Stock solution of olivetol (1 mg/ml):** Dissolve 10 mg olivetol in 10 ml methanol. Ethyl acetate. Methanolic solution of 10% K_2CO_3.

Procedure

(i). **Extraction of alkylresorcinols from cereal kernels:** Place 5 g of whole grains of wheat, barley or rye with 40 ml of ethyl acetate. After 48 h at room temperature, filter the extracts with filter paper. Evaporate the organic solvent to dryness at 40 °C. Then, dissolve methanolic extracts with 2 ml of methanol.

(ii). **Prepare sample tubes:** Place 50 µl of each methanolic extract in tubes. Make up to 200 µl with methanol.

(iii). Prepare fresh working solution of Fast Blue RR reagent: by mixing 1 part of stock reagent with 5 parts methanol.

(iv). Place aliquots of the stock solution of olivetol comprised between 1 and 10 µl (1–10 µg, or 6.3–62.9 nmol) in assay tubes and made up to 200 µl with methanol.

(v). The blank tube only contains 200 µl of methanol.

(vi). Add 2 ml of working solution of Fast Blue RR salt to each tube.

(vii). Add 10 µl of 10% K_2CO_3. Let 15 minutes.

(viii). Determine absorbance at 480 nm against the blank.

Calculations

Step 1. Plot absorbance at 480 nm vs olivetol content. Fit a line function applying regression analysis ($r^2 > 0.80$).

Step 2. Calculate total alkylresorcinol concentration (C) in the sample expressed as olivetol equivalent concentration. Consider absorbance at 480 nm (A_{480}) from sample tube:

$$C\ (\mu g/mL) = A_{480}/\varepsilon$$

Where, ε is the extinction coefficient (slope of the curve) and $C_{olivetol}$ is olivetol concentration. Express in µg of total alkylresorcinols per gram of kernel.

3.5. Resveratrol

Stilbenes are defense biomarkers in *Vitaceae*. They occur as phytoalexins in grapes, leaves and stalks produced in response to biotic (i.e. infection of *Botrytis cinerea*) and abiotic stresses (i.e. aluminium chloride or UV exposure). Resveratrol is the main stilbene from *Vitis* found as glycosides or free in its *trans* form. The rate of synthesis of resveratrol after stress induction depends on the grape variety and provides good measure to evalute the resistance of grapevine cultivars to grey mould and downy mildew (11). Biological (artificial inoculation) and chemical methods (HPLC analysis of stilbenes in grape tissues) have been develop to evaluate the level of resistance of grapevine seedlings to downy and powdery mildew in breeding programmes.

Experiment 5. Analysis of resveratrol in grape leaves and berries

Principle

Trans-resveratrol has an UV-VIS absorption spectra with two peaks of maximum absorbance: from 308 to 336 nm and from 281 to 313 nm. UV maximum absorbance of *trans*-resveratrol is at 308 nm. After separation by a HPLC gradient, resveratrol can be identified and quantified using a diode array detector (2).

Materials and Equipment

Diseases and healthy leaves and grapes of grapevine which are frozen until use. HPLC apparatus coupled to diode array detector and equipped with a C18 column (3 µm, 2 × 150 mm). Liophylizer.

Reagents

Commercial standard of *Trans*-resveratrol. HPLC grade *o*-phosphoric acid, acetonitrile and methanol.

Procedure

(i) **Processing of leaves**: Freeze dry the frozen leaves. Weigh samples of the freeze-dried material (0.25 g) and extract for 30 min in 90% methanol in the dark at room temperature. Then, centrifuge at 3500 rpm for 10 min. Rinse the pellet twice with methanol and Centrifuge again. Pool the supernatants, record total volume and store in a freezing box until the HPLC measurement.

(ii) **Processing of berries:** Proceed with frozen berries as indicated for frozen leaves.

(iii) Filter aqueous-methanolic extracts through a celullose filter membrane (pore size 0.45 µm). Inject 20 µl of the filtered sample in the HPLC coupled to the diode array detector. Mobile phase consists of solvent A (acetonitrile:*o*-phosphoric acid:water, 5:0.1:94.9) and solvent B (acetonitrile: *o*-phosphoric acid:water, 80:0.1:19.9). A gradiente is performed from 0% to 45% of B within 55 min and then from 45% to 100% of B within 10 min. The flow rate is 0.25 ml/min. *Trans*-resveratrol is detected at 315 nm. Resveratrol is quantified by the external standard method.

3.6. Saponins

Saponins are glycosides with a polyciclic triterpenoid or a spirosteroid aglycone (33). Triterpenoid saponins are found primarily in Araliaceae, Leguminosae, Polygalaceae and Cucurbitaceae while steroidal are in the Dioscoreaceae, Liliaceae, and Scrophulariaceae. Most saponins are natural surfactants that produce soap-like foam when they are shaken in water solutions, able to produce hemolysis of blood cells and cytotoxicity on fishes and snails. Several saponins are also effective antimicrobials (23). Legume species such as alfalfa and soybean, and pseudocereals of Chenopodiaceae such as quinoa have been selected for low content of saponins because these secondary metabolites confer a strong bitter taste and restrict availability of proteins, iron and zinc by forming complexes (7). Reduction of saponin content often has meant an increased susceptibility of crops to pests and diseases. Easy and fast bioassays are available for saponin assessment, including the *Trichoderma viridi* bioassay and the hemolytic test (22).

Experiment 6. Determination of saponins in forage species

Principle

Saponins are able to disrupt cell membranes of blood cells due to their surfactant activity. Their content is estimated based on hemolytic activity of plant extracts (24).

Materials and Equipment

Sheep blood, Plant material rich in saponins (i.e. alfalfa leaves), Liophylizer, Rotary evaporator, Analytical Balance (accuracy of 0.01 mg), Microtiter plate.

Reagents

Phosphate buffer saline (PBS) containing 140 mM NaCl, 3 mM KH_2PO_4, 8 mM KCl, and 1 mM Na_2HPO_4 (pH 7.2). Chloroform, methanol, hexane and n-butanol.

Procedure

(i) **Preparation of 3% suspension of red blood cells in PBS:** Take blood from cattle into heparinized tubes containing beads. The beads can be removed soon after taking the blood. Centrifuge the blood at 1500 *g* for 5 min and wash the packed erythrocyte cells three times with the PBS (pH 7.2) by centrifugation and subsequent removal of supernatants. The remaining layer of packed erythrocyte cells is diluted to 3% with the PBS.

(ii) **Preparation of leaf extract:** Ground 10 g of dry leaf sample with the Wiley Mill. Add 10 ml of hexane to the finely ground leaf sample. Left for 2 h and eliminate hexane from the plant material by filtration through filter paper. Left the defatted dry

leaf sample in 50% aqueous methanol for 24 h with occassional hand shaking. Filter the suspension and evaporate the methanol under vacuum at 40 °C in the rotary evaporator. Transfer the aqueous phase in a separating funnel and extract three times with an equal volume of chloroform to remove pigments. Finally extract the concentrated saponins two times in the aqueous solution with equal volume of n-butanol. Evaporate the n-butanol at 40 °C. Dissolve the dried fraction containing saponins in 5 to 10 mL of distilled water and transfer the solution into a separate pre-weighed container. Freeze-dry the fraction and calculate the per cent recovery of saponins.

(iii) Place 50 µl of the red blood cells suspension (3%) in separate wells of a microtiter plate. Dissolve 10 to 20 mg of the freeze-dried saponin-enriched fraction 1 mL of PBS. Prepare a series of twofold diluted solution with the PBS.

(iii) Add a 50 µl aliquot of these twofold diluted solutions of saponins with the PBS to each well and incubate the mixture at room temperature for 2 h.

(iv) At the end of incubation, identify the minimum concentration of the saponin extract that hemolyzes erythrocytes. A clear concentric circle around the red blood cells is indicative of a nonhemolytic well, and the spread of red color in the well and absence of a clear zone around red blood cells shows hemolysis.

(v) The hemolytic activity is expressed as the inverse of the minimum concentration of saponin extract that produced hemolysis. The results can also be referred to the hemolytic activity of a commercial available hemolitic saponin (i. e. saponin mixtures from *Yucca schidigera* or *Quillaja saponaria*) assayed simultaneously with the test sample.

3.7. Phenylalanine ammonia-lyase (PAL)

Phenyl alanine ammonia-lyase (PAL, EC 4.3.1.5) is a key enzyme which allocates significant amounts of carbon from phenylalanine into the biosynthesis of several important secondary metabolites, including antimicrobials (i. e. phytoalexins of flavonoid nature) and structural units needed for lignin synthesis and crosslinking of cell wall components (i.e. ferulic, sinapic and *p*-coumaric acids). PAL catalyzes the non-oxidative deamination of L-phenylalanine to *trans*-cinnamic acid which is the precursor of many phenylpropanoids, viz., lignins, flavonoids and coumarins (6). An increase in PAL is often correlated with higher resistance of crop plants to pests and diseases.

Experiment 7. Assessment of PAL activity

Principle

PAL activity can be measured through production of *trans*-cinnamate since L-phenylalanine. Content of *trans*-cinammate is measured spectrophotometrically at 290 nm

Materials and equipment

Bean seeds, analytical balance, beakers, pipettes, assay tubes, centrifuge tubes, dark bottle, germination paper, mortar and pestle, pH meter (or pH indicator sticks), polypropylene flasks, refrigerated preparative centrifuge capable of speed of 2000 *g,* water bath capable to incubate at 40 °C, ice bath.

Reagents

Nutrient solution: (Prepare a nutrient solution containing 6 mM KNO_3, 3 mM $Ca(NO_3)_2$, 3 mM $MgSO_4$, 1.5 mM KH_2PO_4, 125 µM Fe–EDTA, 10 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 30 nM $(NH_4)_6(Mo7O_2)_4$ and 0.1 µM $CoCl_2$; adjust pH to 5.5); methanol; 1 N NaOH (dissolve 40 g of NaOH in 500 mL of distilled water and make up to 1 L); 0.1 M sodium borate buffer (dissolve 3.8 g of $Na_2B_4O_7$ in 80 mL of distilled water; adjust to pH 8.8 with 1 N NaOH and make up to 100 mL); 50 mM L-phenylalanine (dissolve 0.8 g of L-phenylalanine in 100 mL of distilled water).

Procedure

(i) Bean seedlings: Place the bean seeds in plastic trays (i.e. 55 x 40 x 15 cm) filled with sterile sand. Irrigate with nutrient solution. Place trays in a greenhouse at 25 °C, relative humidity of 80% and 14 h photoperiod. Germinated seeds are left to grow until plantlet stage at which the primary leaves become expanded (about 9 days). Irrigate again during the growth period if needed.

(ii) Induction of PAL activity: Spray leaves with 10 mM salicylic acid with a hand atomizer. Plants sprayed with water will serve as control. After three days, collect leaves from each treatment at random. Weigh the tissue, freeze on dry ice, and store at -80 ºC for later analysis.

(iii) Measure of PAL activity: Procedure for each sample is as under:

(i). Grind 2 g of leaves in a mortar with a pestle, placed on an ice bath, with 4 mL of 0.1 M sodium borate buffer (see Observation 1). Centrifuge homogenates at 2200 *g* for 15 min, containing 0.05 g of polyvinylpyrrolidone, in a blender. Filtrate through a double cheesecloth layer and centrifuge (25,000 *g*) for 15 min.

(ii). Prepare the reaction mixture adding to a tube:
a) 1 mL of sodium borate buffer
b) 0.25 mL of enzyme extract
Pre-incubate for 5 min at 37 °C (see Observation 2).

(iii). Add 0.3 mL of L-phenylalanine to the reaction mixture and incubate for 2 h at 37 °C.

(iv). Read absorbance of reaction mixtures at 290 nm, against a control consisting in the reaction mixture plus 0.3 mL of distilled water.

Statistical analysis

Experiment is designed in a split plot arrangement in a randomized complete design with factors A (cultivars) x B (treatments) and 5 replicates. The sample analyses are done in triplicate and the enzyme specific activities are subjected to analysis of variance. Correlation coefficients may be calculated and means are compared using the Duncan test.

Calculations

Step 1. Calculate concentration of *trans*-cinnamic acid (C) in each sample.

$$C = A_{290} / \varepsilon_{290} \quad (2), \quad \text{where } \varepsilon_{290} = 9630\ M^{-1}\ cm^{-1}$$

For example if A_{290} is 0.35, concentration should be:

$$C = 0.35 / 9630\ M^{-1}\ cm^{-1}$$
$$C = 0.036\ mM\ (or\ 36.34\ nmol/mL)$$

Step 2. Considering the amount Total *trans*-cinnamic acid (tta) content in the assayed volume of tissue extract, considering the total volume (Vte) of tissue extract is known and the assayed volume is 0.25 mL.

$$tta\ (\mu mol) = C\ (nmol / mL) \times Vte / 0.25\ mL$$

Following example in step 1 and considering Vte is 1.55 mL, tta amount should be:

$$tta = 36.34\ nmol/mL \times 1.55\ mL / 0.25$$
$$tta = 225.3\ nmol$$

Step 3. PAL activity is expressed as amount of formed trans-cinnamic acid (nmol/h/g fresh weight):

$$PAL\ activity\ (nmol/h/g\ fw) = [tta\ (nmol/mL) / \Delta t] / weight\ of\ tissue\ (g\ fw)$$

Following the example in step 2, if Δt is 2 h and tissue weight is 2 g:

PAL activity = (225.3 nmol /2 h)/2 g hence, PAL activity = 56.33 nmol/ h/g fw

Observations

1) All material (Pestle and mortar, centrifuge tube) must be refrigerated before enzyme extraction.
2) Make at least three independent experiments (N = 3).

3.8. Salicylic acid

Salicylic acid (SA) is benzoic acid derivative (Figure 6), with signalling functions in local and systemic acquire resistance (SAR) responses (9). Synthesis of SA was an event associated to SAR in several crops because inoculation with pathogens increases SA concentration in both inoculated and non-inoculated plant organs. Current findings suggest that SA is not translocated through the plant and the local increases of SA is due to *de novo* synthesis (25). This also means that an unknown mobile molecule other than SA is responsible for SAR signalling through the non-inoculated plant parts (3). Induction of SA be monitored in breeding programmess as marker of crop resistance to pests and diseases.

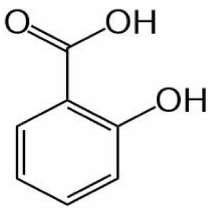


Figure 6. Chemical structure of salycilic acid

Experiment 8. Analysis of salicylic acid

Induction of SA synthesis in plant seedlings can be followed by Thin Layer Chromatography (TLC) methods (29).

Materials and equipment

Sterile syringe and needle, suspension of *Pseudomonas syringae*, cucumber seedlings, 50 µL capillary pipette, centrifuge capable of 10,000 *g*, plates of Silica gel 60 F-254, 1 L glass beaker and a glass piece to cover it (or a TLC tank), long wave UV lamp, analytical balance with accuracy of 0.01 mg.

Reagents

Ethanol; dioxane; ethyl acetate; acetic acid; benzene; salicylic acid solution (add 1 mg of salicylic acid to 10 mL of ethanol).

Procedure

(i) Inoculate cucumber plants with two leaves, by injection into one leaf with cells of a wild type of *Pseudomonas syringae* (recommended level of inoculum: 1 x 10^8 CFU/mL).
(ii) Obtain samples of phloem from cucumber seedlings at different times (6, 12, 16, and 24 h). To do it, cut the ends of petioles of cucumber seedlings. Collect phloem exudates from these cuts with a 50 µL capillary pipette.
(iii) Place known volumes of phloem exudates into three volumes of ethanol to precipitate proteins and other high molecular weight materials.
(iv) Centrifuge at 10,000 *g* for 5 min. Extract pellets with ethanol and combine ethanol extracts.
(v) Evaporate ethanol extract to dryness in a rotary evaporator at 40 °C and dissolve in 50% ethanol, in a volume equal to that original collected as phloem exudates.
(vi) Spot ethanol extract onto a silica gel plate and develop it (see Observation 1). Solvents suggested as mobile phase are toluene: dioxane: acetic acid (90:25:4, v/v), acetic acid: chloroform (1:9, v/v) and ethyl acetate: benzene (9:11, v/v). A control consisting in a salicylic acid standard solution with known concentration is also developed.
(vii) Visualize salicylic under long wave UV light (365 nm, see Observations 2 and 3).

Precautions

Dioxane, ethyl acetate, acetic acid and benzene are toxic solvents. Avoid exposure. Obtain special instructions before use.

Observations

(i). Content of salicylic acid can be measured using a fluorometer. When fluorometer is not available, an alternative is to take a picture of the developed plate and then scan it in a computer for image analysis. NIH image (http://rsb.info.nih.gov/nih-image/) and Scion image (http://www.scioncorp.com/) are public domain image processing programs often used for TLC analysis.
(ii). Salicylic acid can also be visualized spraying with Folin & Ciocalteu's reagent. Dilute the commercial source of this reagent with distilled water in a ratio 1:3 and spray onto the plate. After drying, the plate is also exposed to ammonia fuming to increase intensity. Salicylic acid is visualized as a blue spot.

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