P1 – SAPROV - Marche Polytechnic University, Ancona, Italy

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Topic and contribution

This group is interested to study strawberry germplasm nutritional quality. This Unit will operate in the development and application to the strawberry germplasm the analytical methods commonly defined for the evaluation of fruit quality and nutritional attributes. In particular, fruit from the field experiments will be analysed by testing different methods for detecting fruit total antioxidant capacity, total polyphenol content, anthocyanins and vitamin C, to be correlated also to the standard quality parameters such as firmness (F), colour (C), soluble solid content (SS) and titratable acidity (TA), and fruit size (FZ). The genetic material identified with the most interesting fruit nutritional quality will be used for profiling studies. See annex detailed methods.

Development of in vitro proliferation and conservation protocols (see annex)

This unit will collaborate also to the dissemination activities.

P1 is acting as responsible of Genberry WP4 activities.
WP4. Characterisation of biochemical components linked to health nutritional values (total antioxidants and vitamin C) and evaluation for resistances

The objective is to evaluate and screen large collections, populations and core collections for identifying optimal source fruit quality and of bioactive compounds for conferring health benefits.

<table>
<thead>
<tr>
<th>Components linked to health nutritional values</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
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<tbody>
<tr>
<td>1. Evaluate the collections available by using analytical methods for detecting fruit total antioxidant activity (TAC), and vitamin C.</td>
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<tr>
<td>2. Evaluate the core collections defined in WP1 by using analytical methods for detecting fruit total antioxidant activity (TAC), and vitamin C.</td>
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<td>3. Identification of the genetic material performing with the higher nutritional quality and to be delivered for the high throughput profiling and validation of fruit-derived BC with impact on human diseases</td>
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Description of work

Components linked to health nutritional values
1. Evaluate the collections available by using analytical methods for detecting fruit total antioxidant activity (TAC) and vitamin C.
2. Evaluate the core collections defined in WP1 by using analytical methods for detecting fruit total antioxidant activity (TAC) and vitamin C.
3. Identification of the genetic material performing with the higher nutritional quality and to be delivered for the high throughput profiling and validation of fruit-derived BC with impact on human diseases.
WP4. Characterisation of biochemical components linked to health nutritional values (total antioxidants and vitamin C) and evaluation for resistances

Description of work

Components linked to health nutritional values
1. Evaluate the collections available by using analytical methods for detecting fruit total antioxidant activity (TAC), and vitamin C.
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3. Identification of the genetic material performing with the higher nutritional quality and to be delivered for the high throughput profiling and validation of fruit-derived BC with impact on human diseases

Deliverables

<table>
<thead>
<tr>
<th>Deliverable date</th>
<th>Deliverable description</th>
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<tbody>
<tr>
<td>September 2010</td>
<td>D14. Collections evaluated for health compounds</td>
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Milestones

<table>
<thead>
<tr>
<th>Milestone date</th>
<th>Milestone description</th>
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<tbody>
<tr>
<td>January 2009</td>
<td>List of techniques for evaluating fruit health quality</td>
</tr>
<tr>
<td>September 2010</td>
<td>Characterisation of small berries collections for fruit health quality</td>
</tr>
</tbody>
</table>
P1 - PARTNERS DESCRIPTION

Persons involved: The P1 Geneberry group includes 6 persons among which Bruno Mezzetti, one technician, one post-doc, one PhD student and undergraduate students, which have the knowledge and skills to accomplish the proposed tasks. One more graduate student with a background in biochemical studies will be hired at SAPROV – Marche Polytechnic University for the project, EU funding. SAPROV will also provide the administrative staff requested for the management of the project. The group leader, Bruno Mezzetti, has a 15-year experience on: fruit breeding; horticulture; cell biology; biochemistry, biotechnology of horticultural plants. He has participated to breeding and cell culture projects of strawberry, stone and pome fruits at national and International level. Major achievements produced 20 peer reviewed articles, participations to EU projects (FAIR CT97 3894; FP6 – STREP-FLAVO) and COST Action 836 and COST Action 863, in both cases acting as Chairman of the Action.

Facilities: The Plant Biotechnology and Fruit Quality laboratories at SAPROV Marche Polytechnic University are fully equipped with basic facilities as workbenches, fume hoods, horizontal and vertical flow hoods, growth chambers, walk-in cold room, class 100 clean room, autoclaves, speedvac, incubators, ultrapure water system, etc. Specific instruments in the biochemistry and genetic Lab. are: 1 RealTime PCR Biorad, 96-well Thermocyclers, gel electrophoresis systems, gel documentation stations, bench centrifuges, ultracentrifuges, swing- and fixed-angle rotor centrifuges, spectrophotometers, refrigerators, freezers, etc. The SAPROV department and the Biochemistry Institute also share the following chemical/biochemical apparatus and devices needed in this program (HPLC-UV/VIS or HPLC-EC, spectrophotometers, confocal microscope, fluorescence microscope, ultra-centrifuges, Flow Injection Analysis systems, cell culture laboratories, etc.).
ACTIVITIES RELATED TO THE CHARACTERIZATION OF FRUIT NUTRITIONAL QUALITY OF STRAWBERRY GERMPLASM: DEVELOPMENT OF ANALYTICAL PROTOCOL FOR STRAWBERRY FRUIT EXTRACTION METHOD (see annex detailed protocol)

The extraction starts with the preparation of a solution of methanol and water (80% v/v) to be added to the pieces of strawberry in ratio of 1:5.

10 g of fruit are weighed and used for the extraction.

The extraction consists of two consecutive extractions:

1. First extraction with 20ml of methanol, extraction phase.
2. Homogenize the mixture along 30 minutes. The extraction has to be in dark and covered with aluminum foil.
3. Separate the solid phase from the liquid phase by centrifugation at 4500g for 10 min.
4. Recover the supernatant stock in a falcon tube by a glass Pasteur pipette.
5. Second extraction, from the same fruit, with 20ml of methanol.
6. Repeat the procedures described at points 2. 3. 4.
7. Transfer with a glass pipette the supernatant from the falcon tube to vials and store in freezer at -20°C.

For this type of extraction the determination of Anthocyanin content has to take place immediately after extraction.
ANALYTICAL PROTOCOL FOR STRAWBERRY FRUIT TOTAL ANTIOXIDANT CAPACITY (CAT) by Trolox Equivalent Antioxidant Capacity method (TEAC)

**Chemicals**
- PBS (phosphate buffered saline 5 mM pH 7.2-7.4)
- ABTS stock solution
- ABTS working solution prepared diluting ABTS stock solution with PBS in ratio of 1:50 to 1:70
- Trolox Standard Solution Scale from 0.250 to 2.5 mM (calibration curve)

**Procedure and measuring**
- Supernatant is diluted 1:20.
- The reaction starts after addition of the sample solution respectively blank or standard (100 μL) to 1900 μL of ABTS working solution into the cuvette and should be mixed immediately. The absorbance of the sample is measured after 6 minutes at 734 nm.

**Calibration**
- The Trolox stock solution is diluted with PBS so that the final concentration of the dilution series ranges from 0.025 to 0.450 mmol/l.

**Calculation**
- TEAC - Value (mg Trolox eq/ kg Fruit) = \( \frac{(\Delta A - b) \times F}{a \times E} \)
- \( \Delta A = % \text{inhibition} \)
- \( a = \text{slope} \)
- \( b = \text{intercept} \)
- \( F = \text{dilution factor (20)} \)
- \( E = \text{sample weight (kg/l extracting agent)} \)
ANALYTICAL PROTOCOL FOR STRAWBERRY FRUIT
TOTAL PHENOLICS (FOLIN CIOCALTEU)

Chemicals
- Sodium carbonate solution 20%
- Folin Ciocalteu reagent
- Gallic Acid *Standard solution scale from 10 to 50 mg Gallic acid/l (stored in dark)*

Procedure and measuring
- Supernatant is diluted 1:20.
- The reaction starts after addition of the diluted sample or standard (500 μl) to 3.5 ml of water. Afterwards add 250 μl of Folin-Ciocalteu-Reagent and vortex. After 3 minutes add 750 μl of sodium carbonate and mix one more time. The absorbance of the sample, that must be stored in the dark, is read after 60 minutes at 760 nm.

Calculation

\[
TP \text{ (mg Gallic Acid eq/ kg fruit)} = \frac{(\Delta A - b) \times F}{a \times E}
\]

- \(\Delta A = A_{\text{sample/standard}}\)
- \(a = \text{slope}\)
- \(b = \text{intercept}\)
- \(F = \text{dilution factor (20)}\)
- \(E = \text{sample weight (kg/l extracting agent)}\)
ANALYTICAL PROTOCOL FOR STRAWBERRY FRUIT ANTHOCYANIN CONTENT (PH SHIFT METHOD)

Chemicals
- Buffer pH 1 (potassium chloride 0.025 mol/l)
- Buffer pH 4.5 (sodium acetate 0.4 mol/l)

Procedure and measuring
- Supernatant is diluted 1:10 with each buffer solution.
- The absorbance maximum is determined (about 500 nm depends on fruits variety).
- Each dilution is measured at the absorbance maximum and at 700 nm.

Calculation
Declaration of anthocyanins is given as Pel-3-glu (mg/kg FW) fruit.

\[
\text{mg Pel-3-glu/kg FW} = \frac{[(A_{\lambda_{\text{max}}} - A_{700})_{\text{pH1}} - (A_{\lambda_{\text{max}}} - A_{700})_{\text{pH4.5}}]}{\epsilon \times d \times E} \times \text{MW} \times F \times 1000
\]

- \( A \) = absorbance
- \( \text{MW} \) = molecular weight of pelargonidin-3-glucosid = 433.2 [g/mol]
- \( F \) = dilution factor [-] = 10
- \( d \) = cell pathlengths [cm]
- \( \epsilon \) = molar absorbance of Pel-3-glu = 15600
- \( E \) = sample weight [kg/L extracting agent]
- 1000 = Factor for mg
ANALYTICAL PROTOCOL FOR STRAWBERRY FRUIT 
ASCORBIC ACID CONTENT

- **Chemicals**
  - Oxalic acid solution 2% ig
  - Ascorbic acid standard (it has to be prepared freshly every time)
  - Strawberries sample prepared through a double extraction with a solution of oxalic acid 2% added to the pieces of fruits in ratio of 1:5.
  - Iodide-iodate-solution c(I₂) = 1/128 (it has to be stored darkly)

- **Procedure and measuring**
  - 20 mL supernatant is titrated with iodide-iodate-solution after adding the starch indicator.

- **Calculation**
  - Declaration of ascorbic acid is given as [g / kg] fruit.

  \[
  \text{mg ascorbic acid/kg FW} = \frac{c \times MW \times V}{v \times E}
  \]

  \(c = \) concentration of iodide-iodate-solution [mol/L]

  \(MW = \) molecular weight ascorbic acid 176.13 = [g/mol]

  \(V = \) volume used iodide – iodate [mL]

  \(v = \) volume extract

  \(E = \) sample weight [kg/L extract]
WP4. Characterisation of biochemical components linked to health nutritional values (total antioxidants and vitamin C) and evaluation for resistances

The major aim of WP4 is to evaluate and screen large collections of strawberry and red raspberry for identifying optimal source of fruit quality and of bioactive compounds for conferring health benefits as is confirmed in

Task 1 - Components linked to health nutritional values

- Sub task 1.1 Evaluate the collections available by using analytical methods for detecting fruit total antioxidant activity (TAC), and vitamin C.

For the first year (2007) were identified the following activities:

- Definition of analytical protocols for the bioactive compounds (Total Antioxidant Capacity-TAC, Total Phenolics-TP, Total Anthocyanin Content-ACY, and Vitamin C Content).
- Identification of a first list of accessions available in the different collections to be used for a comparative analyses.
- First application of the analytical protocols for the characterization of a selected number of accessions.
First year data related to the characterisation of biochemical components linked to health nutritional values (total antioxidants and vitamin C) and evaluation for resistances

- Sub task 1.1 Evaluate the collections available by using analytical methods for detecting fruit total antioxidant activity (TAC), and vitamin C.

For the first year (2007) the partners those confirmed their availability to be involved in this study and to provide a list of material available in their collections were been:

- P2 – CRA
- P4 – LIH
- P5 – CIREF
- P6 – BAZ – IOZ
- P8 – IFAPA

In the first year the analytical protocols were have been tested on fruit of 47 accessions derived from 4 different collections: Villanave d’Ornon (CIREF, Creation Varietale Fraises Fruits Rouges-France), Azienda didattico Sperimentale “P. Rosati” Agugliano (AN-Italy), Martorano 5 Forlì-Cesena (Italy), Lithuanian Institute of Horticulture Babtai (Lithuania). In particular:

- **21 accessions from P1 Ancona collection**: Anita, Roxana, Corona, Elsanta, Clery, Ciflorette, Evangeline, Antea, Daroryal, Candonga, Darsilette, Asia, Record, Darselect, Patty, Queen Elisa, Sveva, Dora, Alba, Adria, Irma, Onda
- **14 from P2 – CRA collection**: Candonga, Ciflorette, Clery, Darisette, Daroyal, Darselect, Onda, Queen Elisa (as “new” genotypes), Annalie, Regina, Tardiva di Romagna, Louis Gauthier, Avalon Classic, Rabunda (as “old” genotypes).
- **11 from P5 – CIREF**: Charlotte, Ciflorette, Cirafine, Clery, Darselect, Docteur Morere, Louis Gauthier, Madame Moutot, Mara des bois, Ostara, Rabunda
- **1 from P4 – LIH**: Dange
First year data related to the characterisation of biochemical components linked to health nutritional values (total antioxidants and vitamin C) and evaluation for resistances

- **Sub task 1.2 Evaluate the core collections defined in WP1 by using analytical methods for detecting fruit total antioxidant activity (TAC), and vitamin C.**

During the second year (2008) were been identified what partners will be involved in performing the evaluation of fruit nutritional quality and what type of analyses that will be performed by each partner on their collection:

- P1 Ancona: total antioxidants capacity, total polyphenols, anthocyanins and vitamin C
- P2 – CRA: Total antioxidants capacity + Vitamin C
- P6 - BAZ – IOZ: Vitamin C
- P8 – IFAPA: total polyphenols + flavonoids + anthocyanins

Each partner also drafted the list of varieties to be analysed for the next year.

- **Sub task 1.3 Identification of the genetic material performing with the higher nutritional quality and to be delivered for the high throughput profiling and validation of fruit-derived BC with impact on human diseases**

For the third year of project (2009) will be identified a more detailed study and identification of the genetic material performing with the highest nutritional quality and to be delivered for the high throughput profiling and validation of fruit-derived BC with impact on human diseases
RESULTS P1 – AN
CAT (mg Trolox eq/kg FW)
TP (mg Gallic Acid/kg FW)
ACY (mg Pel-3-glu/kg FW)
RESULTS P2 - CRA
CAT (mg Trolox eq/kg FW)
TP (mg Gallic Acid/kg FW)
ACY (mg Pel-3-glu/kg FW)
RESULTS P5 – CIREF
CAT (mg Trolox eq/kg FW)
TP (mg Gallic Acid/kg FW)
ACY (mg Pel-3-glu/kg FW)
RESULTS P4 – LHI
CAT (mg Trolox eq/kg FW)
TP (mg Gallic Acid/kg FW)
ACY (mg Pel-3-glu/kg FW)
RESULTS: CAT (mg Trolox eq/kg FW) between accessions from P2 - CRA and P1 - AN
RESULTS: TP (mg Gallic Acid/kg FW) between accessions from P2 - CRA and P1 - AN
RESULTS: ACY (mg Pel-3-glu/kg FW ) between accessions from P2 - CRA and P1 - AN
RESULTS: CAT (mg Trolox eq/kg FW) between accessions from P2 – CRA, P1 – AN and P5 - CIREF

CAT (mg Trolox eq/kg FW) between accessions from P2 - CRA, P1 - AN AND P5 - CIREF

[Bar chart showing CAT values for different accessions, with error bars for each bar]
RESULTS: TP (mg Gallic Acid/kg FW) between accessions from P2 – CRA, P1 – AN and P5 - CIREF

![Graph showing TP (mg Gallic Acid/kg FW) between accessions from P2 - CRA, P1 - AN AND P5 - CIREF]
RESULTS: ACY (mg Pel-3-glu/kg FW ) between accessions from P2 – CRA, P1 – AN and P5 - CIREF
RESULTS: CAT (mg Trolox eq/kg FW) between accessions from P2 - CRA and P5 - CIREF

CAT (mg Trolox eq/kg FW) between accessions from P2 - CRA AND P5 - CIREF

LOUIS GAUTHIER

RABUNDA

P2 - CRA  P5 - CIREF
RESULTS: TP (mg Gallic Acid/kg FW) between accessions from P2 - CRA and P5 - CIREF

![Graph showing TP (mg Galli Acid/kg FW) between accessions from P2 - CRA AND P5 - CIREF](chart.png)
RESULTS: ACY (mg Pel-3-glu/kg FW) between accessions from P2 - CRA and P5 - CIREF