SUMMARY OF WORKS

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A grafted plant, during development, undergoes a series of anatomical, physiological and biochemical changes. After grafting, there is a welding process in which the injured areas of the graft and rootstock develop an undifferentiated parenchymatous tissue, called callus. This tissue tends to cover both surfaces coming into contact, as well as the gap between them. After callus formation it follows the generation of new vascular tissues (xylem and phloem) that will restore the ability to transport water and nutrients between the two partners, which now can be called symbionts.

The rapid welding and vascularization of the graft site depends on the degree of compatibility between the two partners. If partners are incompatible layers of cambium and felogen and later of suber are generated between the two surfaces. This process will scar the injured areas, isolating each of the two partners which means that the welding and vascularization processes will not occur. Between the two extreme cases there are many intermediary situations. For example, the initial welding process may take place, but due to the low degree of compatibility, may appear different symptoms such as: lack of grip on grafting, swelling and uneven thickening in the grafti ng point, poor vegetative growth, that will cause premature drying and separation or dis continuity at the graft site. In addition, undifferentiated parenchyma welding line, vascular tissue deformation, distortion, discontinuity of wood and bark, wood decks formation, abnormal distribution of starch, may also appear.

Typically, the graft incompatibility is detected when there are visible symptoms (Hartmann et al. 1997) or anatomical abnormalities at the grafting site. The time required to develop such symptoms is usually very long, and in some cases can take years. In addition, anatomical symptoms manifested in the early stages of tree development, may not always be clearly associated with the phenomenon of incompatibility (Andrews and Marguez, 1993). Thus, developing methods to detect a priori the incompatible combinations would help to reduce the economic losses in the fruit tree growing practice.

Although it is clear that the phenomenon of incompatibility is determined by genetic differences of the two part ners the mechanism of graft rejection phenomenon is still poorly understood. There is a number of hypotheses trying to explain the cause of the phenomenon in terms of growth and development of symbionts, physiological and biochemical differences between scion and rootstock, water restrictions at the grafting site (Kolb and Sperry (1999), Hack et al. (2000)) but none of them is fully reasoned and supported by relevant experimental data.

Experimental research showed that the artificial manipulation of the grafting process may negatively affect the water conductivity (Sperryet al., 1993; Saliendra et al., 1995) which will limit the transpiration rate (Pokman and Sperry, 1993) and carbon assimilation (Hubbard et al., 2001). Some rootstock may significantly influence the growth rate of grafted trees (Rogers and Beakbane, 1957, Lockard and Schneider, 1981 Webster, 1995) through various mechanisms such as influence on the hormonal balance (Kamboj et al., 1999), mineral nutrition (Jones, 1971) and fluid regime (Oliena and Lakso, 1986).

Theoretically, any cell tissue, under optimal conditions, can be totipotent and regenerate entire plants. In vitro cultivation requires the separation of tissue fragments from the complex of factors that control the whole plant. But this is not sufficient to induce division and cell proliferation (meristematic behavior). In order to induce multiplication, cell explants inoculated on sterile medium, must undergo a process of conversion to the meristematic state. This process is called dediferentiation and involves the initiation at the cellular level of changes that would lead to the restoration of the functional characteristics. The plasticity, which means the ability of a genotype to regenerate in function of the culture conditions is particularly high in plant tissues, since most of them are not deeply specialized (such as animal tissue), and can differentiate gradually. Although the organization of cells in tissues or organs suppresses the totipotency, this will be expressed once the cell is detached from the particular structures and follow its own path to restore the previous structures. Growth hormones (auxine, citokinine, gibberellinic) added in the culture medium single or in combinations can determine the type of growth and differentiation, resulting either a mass of cells that proliferate anarchic or primary roots, shoots or somatic embryos.

In general, the sequence of events occuring during in vitro dedifferentiation and regeneration is pretty similar to that taking place under the traditional methods of vegetative multiplication. From this point of view, one can conclude that in vitro cell and tissue culture techniques can be used to produce faster biological material that the traditional methods. This method is also advatageous for the minimizing of environment influence on the biological material characteristics, obtaining of a large number of individuals genotypically uniform in a small space, throughout the year, accelerating growth and regeneration, etc.. Besides these, the applications of plant biotechnology techniques (biochemical markers, DNA analysis, biotransformation) can be much easier. In recent decades a particular interest has been granted to the possibility of in vitro cultivation of temperate zone fruit species. The use of meristem culture led to the development of micropropagation techniques as an alternative to traditional methods of propagation. On the other hand many wood species were found to be unable to regenerate whole plants from plant tissue cultures. For recalcitrant species, in order to obtain virus free plants, Murashige and his colleagues have developed a technique called in vitro micrografting. This technique began to be increasingly used for various purposes such as micropropagation, production of pathogen free plants, and also to elucidate some issues of incompatibility phenomenon manifested in various fruit species. One of the pioneers in implementing this technique in citrus was Navarro (1975) but also important results have been obtained for other species such as Citrus spp, Malus pumila L. Prunus armeniaca L. Prunus amygdalus L. (Yeomann, MM, Kilpatric DC, Miedzybrodzka, MB, Gould, AR 1978, Parkinson, M., Yeomann, MM 1982, Jeffree, CE, Yeoman, MM 1983, Navarro, L. 1988). Parkinson and Yeoman perfomed anatomical and morphological studies of in vitro micro graft while Cantor described in details the anatomical and morphological changes in Vitis vinifera L. Later this technique was successfully applied to elucidate some classical problems of scion-rootstock incompatibility such as the production of polyphenolic substances and resins (Joley et Opitz, 1971, IM -Barazi et Schwabe, 1982; Sheibani et de Villiers, 1995, Jonard et al., 1988) or rejuvenilization (Revill et al., 1996, Estrada -Moon et al., 2002).

Micrografting requires the placing on aseptic conditions of a millimeter-sized piece of graft (0.8-3mm) over a rootstock produced in vivo or in vitro. The resulting plant can be further grown in vitro, or naturalized. Although the technique was tested on many plant species the results can partly be extrapolated to other species because the optimal conditions for production and subsequent cultivation of a certain plant species are highly specific. However, going over the differences it can be concluded that the micrografting techniques involve some common steps as described below.

Preparation of the rootstock:

Most rootstocks are produced by aseptic germination of seeds. These are chemically sterilized using agents such as ethanol 80%, sodium hypochlorite, then washed several times with sterile distilled water. Germination can take place both on filter paper soaked with sterile distilled water and solid (agar) medium. Manipulation is done with sterile instruments in a laminar flow box. The most often used culture media are simple, with no added sucrose or phytohormones, and germination takes place in growth chambers under conditions of optimum temperature for each species, usually 25 ° C in the dark. The cotyledons are cut off from the seedling which is further used as rootstock.

Preparation of grafts.

As graft it can be used any harvested crop sprouts produced in vivo or in vitro through various techniques of micropropagation. The detached apical meristem is sterilized by immersion in 70% ethanol solution for several seconds and after in 12% sodium hypochlorite for 15 minutes. After sterilization it is placed on a culture medium which is usually MS medium with various hormonal balance depending on the species used and maintained for a month. When it reaches a size of 3 to 15 mm it can be used for micrografting.

Grafting technique:

Rootstocks are incised longitudinally and the micrograft cut as „V" shape is inserted into this incision

In vivo growing of the micrograft:

The micrografted plantlets are subsequently maintained in vitro on different media cultures, with low or zero phytohormones. They are kept for one week in low light and higher humidity and after the temperature and light intensity are increased to the optimum for that species untip lateral shoots are formed.

Transfer to soil:

After 10-12 weeks of in vitro culture the plants can be transferred to the external environment, with all the precautions used when transferring any type of plants obtained by micropropagation.

Based on the data from literature, we planed in 2008 to conduct experiments to develop a technique in order to achieve optimal in vitro micrografting using varieties with different degrees of compatibility with quince. Both apical and lateral micrografting with micrograft of different origins, sizes in sterile and non sterile conditions has been tested. The optimal conditions for transfer of the plants in soil have also been established. To achieve these aims, during this period equipment , instruments and glassware, materials and reagents needed for the preparation of culture media have been purchased and the methods and techniques for in vitro cultivation have been established. Recent research in this field indicates that the phenomenon of incompatibility can not be reduced only to the morpho-physiological changes occurring at the point of grafting. There are also changes in the metabolic processes. The involvement of enzymes (catalase, peroxidase, phosphatase) in the process of incompatibility of grafted trees was studied by many authors (Quesada and Macheix, 1984, Delo and Hebant, 1982, Schmidt and Feucht, 1985, Fernandez-Garcia et al. 2004), but their action is not yet fully elucidated. Numerous studies revealed the involvement of various chemical compounds in the process of fixing the graft. Polyphenols play an important role in forming the graft union between partners by involving them in processes of tissue lignification (Haslam, 1979). Also a number of studies have been perform in order to establish biochemical markers for the incompatibility phenomenon. Lachaund (in 1975) suggests that the incompatibility of partners can be avoided by choosing partners with similar protein composition. Comparisons were made between the protein profiles of Prunus species in order to detect the incompatibility before grafting (Huang et al., 1984, Schmid and Feucht, 1985). The content of total soluble proteins in different combinations scion/rootstock characterized by different level of compatibility was exmained by Moreno et al. (1994). They detected a lower amount of soluble proteins in incompatible than compatible combinations.

Although the cell necrosis at the contact of scions with the quince rootsock caused by hydrogen cyanide was demonstrated since 1968 by Gur was and his colleagues the molecular process underlying grafting still remains unknown. The most widely used biochemical markers for different purposes are the isoenzymes. Their use is determined by the fact that they have a high stability against environmental conditions, expresscodominance and show a good reproducibility. Their genetic determinism is associated with the existence of a very few variable loci, which can be easily analyzed by conventional enzymatic methods. Since 1982, Santamour has shown the significance of isoperoxidase pattern for the taxonomic classification of wood species. They can be an important biochemical marker of incompatibility phenomenon because they are involved in the lignification that is essential to achieve a strong weld at the grafting point. The isoperoxidases mediate the polymerization process of lignin and cinnamic alcohol that also participate in the carbohydrate processing. Given these considerations, a comparison of the isoperoxidase pattern of scion and rootstock can provide a starting point for elucidating the causes of lack of affinity to grafting. To determine this pattern, the method of Gulen et al (2002) can be used as described below.

Plant samples are grinded and extracted with an extraction buffer consisting of 0.1 M potassium phosphate, pH 7.5, 30 mM boric acid, 50 mM L-ascorbic acid, 17 mM sodium metabisulphite, 16 mM dithiocarbamate acid, 1 mM EDTA, and 4% (w / v) PVP -40 and the final pH is adjusted to 7.5 with NaOH. The homogenate is centrifuged at 16000g, 30 minutes and 4 ° C and the supernatant is used for electrophoresis.

The electrophoretic separation is carried out at 20 mA for 30 min, then at 40 mA for 3 hours. Gels are stained for peroxidase using the method of Weedwn Wendel (1989), then washed with distilled water, fixed and keep in 10% glycerol. The relative distance of migration of bands (Rf) is calculated after Manganaris and Alston (1992).

This method has been tested by us in order to determine the isoperoxidase pattern of the pear scion and quince rootstock used for grafting. The data will be correlated with morphometric and biometric determinations to elucidate the influence of differences between the pattern of isoperoxidase in graft and rootostock on the grafting process.

Possible implication of polyamine in compatibility was also studied. The polyamines are involved in many processes of growth and development in higher plants as radical oxygen scavengers (Bouchereau, 1999) or as growth regulators (Bajaj and Rajam, 1996; Tanq et al., 2004). Most common forms are putrescine (butane-l ,4-diamine), spermidine [N-(3-aminopropyl) butane-l ,4-diamine] and spermine [NN'-bis-(3 - aminopropyl) butanol, 4 - diamine]. They can be easily determined both quantitatively and qualitatively by HPLC technique (Kotzab asis et al., 1993).

Although their role in stress has been widely investigated, their involvement in tissue regeneration is still poorly understood. On this context we proposed to test the pattern of polyamines during the development process of welding n different combinations with different degree of affinity.

To determine their pattern polyamines are subject to acidic hydrolysis using the method of Flores and Galston (1982). The supernatant obtained after the centrfugation of samples is used for the HPLC analysis.

Standard solutions of polyamines are prepared in eight weeks and stabilized at 4 °. Using successive dilutions a calibration curve has been established.

Amino compounds were determined by HPLC using the method of bauzi Blaise et al., 1995. 20 ml of digested plant sample are mixed with 50 ml borate buffer pH 8.5 and 100 ml chloroform fluorenilmetil-8 mg / 1 in acetonitrile. After 3 minutes, 50 ml of 0.55 M ammonia solution and 300 ml of solution consisting of $CH_3CN/CH_3COOH/H_2O$: 20/2/3 are added. The amino compounds are separated into Superspher 100 RP 18, 125 mm x 4 mm column 5 mm, and read in spectrophotometer at λexc. 263 nm, λem. 313 nm.