COMPARISON OF DIFFERENT TECHNIQUES FOR INOCULATION OF "*CANDIDATUS* PHYTOPLASMA MALI" ON APPLE AND PERIWINKLE IN BIOLOGICAL INDEXING PROCEDURE

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SUMMARY

As phytoplasmas are non cultivable micro-organisms, the research on phytoplasmal diseases can only be achieved with infected hosts. Biological indexing (by grafting) is the simplest detection method for phytoplasmal diseases. We tested four different grafting techniques for inoculation of apple trees or periwinkles in greenhouse, including whip graft, bark graft, budding and chip-budding. All techniques were tested on apple trees (six trees per phytoplasma isolates) in insect-proof greenhouse. The whip and bark grafting were not feasible for periwinkle plants, because of fineness and fragility of their tissues: only the chip-budding was performed (four plants per isolate). In apple trees, the best and soonest positive results were obtained by chip and bark grafting. Except for seven transplants not-grown after grafting, 100% efficiency of inoculation was obtained by both methods. Nevertheless, the transmission of phytoplasma from transplant not-grown to rootstock was sometimes recorded (28.6%). The earliest phytoplasma symptoms after whip or bark grafting appeared after 3 months. Symptoms were obtained much later with budding and chip-budding. In case of periwinkles, infected apple and periwinkle materials were used as inoculum sources. Transmission of phytoplasma from periwinkle to periwinkle was successfully carried out by chip-budding grafting. The symptoms were observed during the second month after inoculation. The transmission of phytoplasma from infected apple material to periwinkle (by chip-budding) was achieved for 60 % of the tested samples. Moreover, the latency period before symptom observation was longer. Finally, we perceived the apple trees are more convenient and rapid than periwinkle plants for biological indexing of apple materials.

Key words: phytoplasma, Apple proliferation, biological indexing, grafting, certification.

INTRODUCTION

Phytoplasmas are prokaryotes belonging to *Mollicutes* class since they lack a cell wall, a group of organisms phylogenetically related to low G+C content Gram positive bacteria. Phytoplasmal plant diseases are spread by sap-sucking insect vectors (Lee *et al.*, 2000). They inhabit phloem of hundreds of plant species, are responsible of diseases spread worldwide and, in several cases, associated with severe epidemics of very often quarantine importance. Phytoplasma infected plants show symptoms such as yellowing, witches' broom, virescence, phyllody, leaf roll and decline. Apple proliferation (AP) disease, caused by "*Candidatus* Phytoplasma mali" ("*Ca* P. mali"), is one of the most important apple diseases affecting both the fruits yield and quality. It belongs to the EPPO A2 list of quarantine plant pathogens.

These micro-organisms are obligatory pathogens and, up to now, they were not cultivated in axenic culture; therefore Koch postulates are only sometimes fulfilled by using alternative tools, such as graft or insect transmission (Bertaccini, 2007). In other hand, their accurate detection is a major prerequisite to control the disease and fulfil certification requirements. So, phytoplasma graft transmission remains of outstanding importance in disease detection and in the phytosanitary certification schemes.

Different grafting methods, depending on scion and rootstock characteristics, may be used on woody or herbaceous plants to detect phytoplasma infection. There are many methods of grafting (whip, bark or side grafting, budding and chip-budding) which differ only in detail of technique. Sometimes one method is superior for some particular purpose or occasion. Regardless of the method used, the principles involved remain constant. To be successful at grafting, understanding a few basic points about tree anatomy will be important. The cambium is a thin layer of cells that lies between the bark and the wood. When the bark is peeled off a tree in the spring, the cambium is the slippery layer that separates. This is the growth layer on the tree or stock receiving the graft that must come in contact with the same layer on the piece to be grafted on, or scion. The cambium layer and resulting callus growth is very easily dried out and destroyed. Grafting compound and rubber budding strips should be used to prevent drying out.

In this study, for the first time, comparisons between 4 grafting techniques on apple and periwinkle plants for inoculation of "*Ca* P. mali" and its detection by biological indexing are presented.

MATERIALS AND METHODS

Healthy and infected plant materials

Healthy MM106 apple rootstocks and AP-infected scion woods (5 isolates) were provided by CRA-W (Gembloux, Belgium) and Quarantine Station (Lempdes, France), respectively. Healthy and AP-infected (5 isolates) periwinkles were prepared by FUSAGx and Institut für Pflanzenschutz (Dossenheim, Germany), respectively. MM106 rootstocks are very susceptible to Apple proliferation phytoplasma (Jarausch *et al.*, 1996).

All inoculation experiments were carried out in July-August in insect-proof greenhouse (14h light, high relative humidity, 20-25°C).

Whip (tongue) graft

The whip (tongue) graft method works best when the stock and scion are of similar diameter, preferably between 8 and 12 mm. For manipulation, a branch of the under-stock was cut off, leaving a stub about 15-30 cm long. A straight, slanting cut about 3 cm long on both the scion and the stock was made (Figure 1A). For the tongue, a straight draw cut was made (not split), beginning near the top and cutting about the full length of the level (Figure 1A). The two parts were matched together (Figure 1B) (Hertz, 1993).

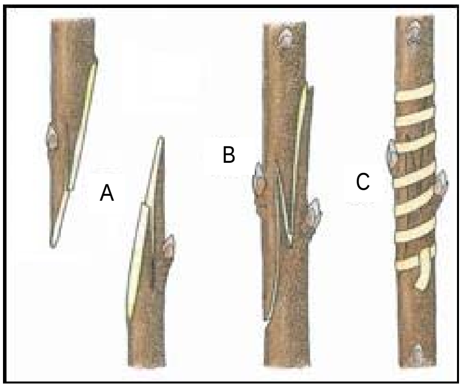

Figure 1. Whip (tongue) grafting schema
(borrowed from www.gartenbauvereine.org/texte/merkinfo/m_VeredelungObst.html)

Bark (veneer) graft

Bark grafting may be performed on branches ranging from 12 to 25 mm in diameter. The bark graft can be made only when the bark slips or easily separates from the wood. Branches of large trees or the trunk of a small tree must be sawed off to provide a stock for the scions. Two techniques can be used on the stock for the bark graft: making a slit in the bark about 2 cm long (Figure 2A left), or making two slits in the bark separated by the width of the scion (Figure 2A right). The scion should be 5 to 12 cm long with two to three buds. The base of the scion was prepared by cutting inward 2 to 5 cm from the base then downward, forming a shoulder and long, smooth cut (Figure 2B). The scion was pushed down in the slit or between both slits if the double slit method is used (Figure 2C) (Weinmann, 2002).

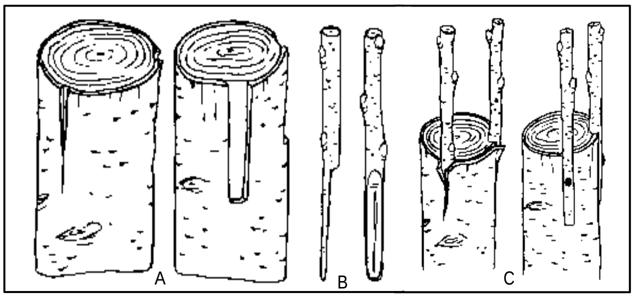

Figure 2. Bark (veneer) grafting schema (Weinmann, 2002)

Budding

Budding is a form of grafting in which a single bud with a thin layer of bark is used as the scion rather than a section of stem. Budding is carried out in summer, usually from July 15 to August 15, when the bark of the stock slips easily and when there are well-grown buds. The first step is to cut bud sticks of the desired cultivar from strong shoots of the present season's growth (Figure 3A). On the branches of the stock, about 30-50 cm or more from the trunk, a T cut was made just across the bark (Figure 3B). The bud is put under the flaps (Figure 3C) (Hertz, 1993).

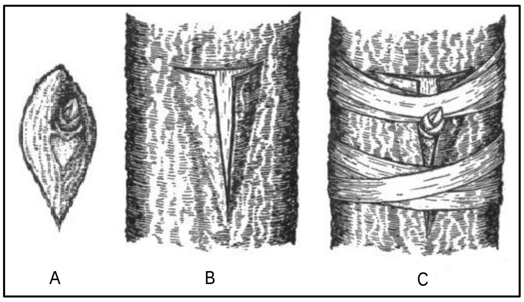

Figure 3. Budding grafting schema
(borrowed from www.gutenberg.org /files/18189/18189-h/18189-h.htm)

Chip-budding

For chip-budding, in mid-summer, non-flowering shoots of rootstocks are selected with similar diameter to the scions from well-ripened, current season's growth as bud. Using a clean sharp knife, a cut is made 2 cm below a bud, inserting the blade about 5 mm deep at an angle of 30 degrees. A second cut is made about 4 cm above the first and cutting continued down through the wood to meet the first cut (without damage to bud) (Figure 4C and D). Two cuts are made in the rootstock about 15 cm from the ground to correspond with those on the bud chip and the resulting sliver of wood is removed (Figure 4A and B). The bud chip is placed into the 'lip' of the cut rootstock so that the cambium layers are matched (Figure 4E) (Anonym, 2005).

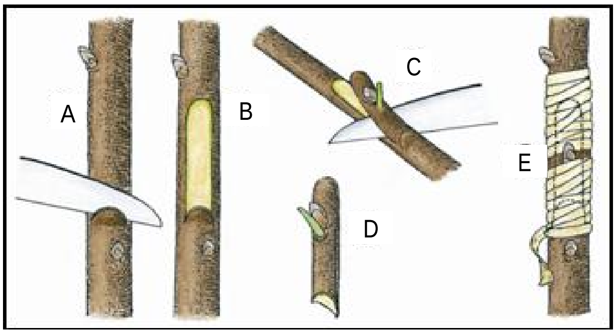

Figure 4. Chip-budding grafting schema
(borrowed from www.gartenbauvereine.org/texte/merkinfo/m_VeredelungObst.html)

In all grafting techniques the union (rootstock-scion) was always bound tightly with tape and, for whip and bark grafting the union and scion were carefully covered with plastic bag for 1-2 weeks. The symptom emergence was monitored on inoculated plants by time. In periwinkle plants, the symptoms as yellowing and reduced leaf size and/or vigour were surveyed. In apple trees, proliferation, chlorosis, small leaves and enlarged stipules were noticed.

RESULTS

Biological indexing on periwinkle plants

Because of fineness and fragility of their tissues, the whip and bark grafting were not feasible. Budding have not previously been tested on periwinkle. So, only the chip-budding was performed. In this case, we used previously infected apple and periwinkle materials as the source of inoculum. Transmission of phytoplasma from periwinkle to periwinkle was successfully documented for more than 90% by appearance of symptoms during the second month after grafting (Table 1). The surprising transmission of phytoplasma from infected apple material to periwinkle was achieved for 60 % of the tested samples (Table 1), but the latency period before symptom observation was notably longer (4-6 months) than those inoculated by infected periwinkles. To our knowledge, this is the first report of AP transmission from apple to periwinkle.

Biological indexing on apple plants

In this case, all grafting methods were performed. The best and soonest results as symptoms appearance of phytoplasma inoculation was recorded with whip and bark grafting methods. An efficiency of 100% of inoculations was obtained by whip and bark methods (Table 1). In our tests, 7 transplants of whip or bark grafting were not covered by bag, so they did not grow after grafting. Nevertheless, the phytoplasma transmission from them to the rootstock was sometimes recorded (28.6%). The earliest phytoplasma symptoms by whip or bark grafting appeared after 3 months. Also, the efficiency of budding and chip-budding were 38 and 54%, respectively (Table 1), and symptoms were observed much later (normally in next year) with these grafting methods.

Table 1. Biological indexing of "*Ca* P. mali" by different grafting methods

Grafting method	Periwinkle* inoculated by		Apple* inoculated by
	Periwinkle materials	Apple materials	Apple materials
Whip graft	-	-	10/10
Bark graft	-	-	11/11
Budding	-	-	3/8
Chip-budding	9/10	12/20	6/11

* : symptomatic plants number per total tested plants.
- : not-tested.

DISCUSSION

Despite disadvantages of biological indexing as being laborious, time-consuming, and skill-demanding (Di Terlizzi, 1998), and even if the molecular and serological diagnostic protocols can replace biological indexing as a fast screening technique in certification programs, biological indexing will often remain mandatory as a second screening technique to fulfil the Koch's postulate within the sanitary programs and certification of propagate materials. Even with the advances in new technologies for improved diagnostic methods, many actual works show that the biological indexing remains the base of a certification program. In this context, our work allowed the identification of the best suited methodology for AP indexing. The best results were obtained with whip and bark grafting on MM106 rootstocks, and we perceive apple trees are more suitable than periwinkle for biological indexing of apple materials, if the graft season would be suitable in greenhouse. Finally, within a certification program, symptom observation after biological indexing could be confirmed by phytoplasma detection by means of molecular methods.

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