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"Population dynamics of Apple Proliferation in Trentino"

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To who belives in the dreams …

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SOMMARIO

Apple Proliferation (AP) è una grave malattia del melo diffusa in tutti i paesi frutticoli d'Europa causata dal fitoplasma *Candidatus* Phytoplasma mali. Questo organismo è un procariote della classe Mollicutes che mostra pleomorfismo, cioè variazioni di forma, per la mancanza di parete cellulare. A differenza degli altri mollicuti non è coltivabile *in vitro*.

Le piante infette mostrano una vegetazione apicale affastellata, simile a scope a causa dall'anticipato germogliamento delle gemme laterali e successiva emissione di getti. Per questo la malattia è nota anche come "Scopazzi del melo". La colorazione anticipata in tarda estate e la produzione di frutti piccoli e di valore organolettico nullo sono altri sintomi primari della malattia. Si ha quindi la perdita del raccolto con notevoli ripercussioni economiche.

La presenza dell'infezione in Trentino è accertata fin dal 1950 manifestandosi con incidenza variabile. Particolarmente in questi ultimi anni l'entità dell'infezione è in continua progressione interessando le varietà più significative come *Golden delicious*, *Renetta del Canada*, *Gala* e le varietà del gruppo delle delicious rosse su portainnesti di diversa vigoria. Tutte le varietà ed i portinnesti del melo validi commercialmente sono suscettibili alla malattia.

Il patogeno è diffuso da 2 specie di psille, *Cacopsylla picta* e *Cacopsylla melanoneura*, insetti dell'ordine degli Omotteri, dotati di apparato boccale di tipo pungente-succhiante in grado di penetrare nelle cellule floematiche e di succhiare la linfa elaborata.

Alcuni esperimenti hanno dimostrato che nelle condizioni locali *C. picta* gioca un ruolo nella trasmissione di AP alle piante di melo mentre *C. melanoneura* è risultata finora solo ospite del patogeno.

La malattia è trasmissibile anche attraverso tutte le forme di moltiplicazione agamica del melo (innesto compreso). Si ipotizza che la diffusione del patogeno possa avvenire anche mediante il passaggio dei fitoplasmi tramite anastomosi di radice. Finora non sono noti mezzi di lotta in grado di contrastare le fitoplasmosi.

Per definire la filogenesi e un'accurata classificazione dei fitoplasmi è stata utilizzata l'analisi del gene 16S rDNA, definito come marcatore filogenetico universale. In base a questo esame è risultato che il fitoplasma di AP presenta una stretta relazione filogenetica con altri fitoplasmi dei fruttiferi europei come l'European Stone Fruit Yellows (ESFY), noto come

giallumi delle drupacee, ed il Pear Decline (PD), conosciuto come moria del pero. Per questo sono tutti classificati in un unico gruppo, detto gruppo AP o 16SrX.
Da qualche anno sono stati determinati 3 ceppi del phytoplasma che si differenziano per 2 mutazioni puntiformi (Jarausch *et al.*, 2000): i ceppi AT1, AT2 e AP.
Nello studio di questa problematica si inserisce questo lavoro con lo scopo di descrivere la struttura e la dinamica della popolazione di questo patogeno, in modo da contribuire a comprendere meglio l'epidemia causata dal fitoplasma. Questo lavoro è parte del progetto SMAP che porta avanti accurate indagini e ricerche su questa malattia, interessando molteplici settori tra cui l'analisi delle condizioni ambientali nelle aree coltivate e la ricerca di materiale vegetale resistente partendo da portainnesti apomittici.
Un secondo obiettivo è la ricerca di eventuali altri insetti vettori del fitoplasma, verificando innanzitutto la presenza del patogeno nell'insetto e successivamente la loro capacità di trasmissione. La ricerca si è focalizzata sugli afidi poiché sono ampiamente diffusi nelle coltivazioni di melo. Gli afidi sono noti per essere vettori di virus, ma non di fitoplasmi.

L'analisi della variabilità genetica del fitoplasma si è svolta sia su materiale vegetale sia su insetti vettori.
Nel campionamento delle piante sono state considerate alcune variabili quali il tipo di cultivar e di portainnesto, l'età della pianta e la zona di provenienza del campione.
Gli insetti sono stati raccolti periodicamente nei frutteti e suddivisi per specie, prima di determinare la presenza del fitoplasma. Le psille sono state suddivise anche per stadio di sviluppo. Le psille *C. picta* e *C. melanoneura*, e gli afidi *Aphis pomi* (Afide verde), *Dysaphis plantaginea* (Afide cenerognolo) e *Eriosoma lanigerum* (Afide lanigero) sono le specie più frequenti.
Il Trentino è stato suddiviso in 10 aree di raccolta, 5 situate nelle valli di nord-ovest, dove l'incidenza dell'infezione è elevata e la coltivazione del melo è di tipo intensivo, e 5 nelle valli del centro-sud, dove la coltivazione è meno diffusa e la presenza del patogeno è ridotta (zone di bassa incidenza).
Al secondo anno di lavoro, oltre alle 10 aree, il campionamento ha interessato 3 micro-aree, che nell'anno precedente presentavano una particolare distribuzione dei ceppi, e 2 micro-aree recentemente bonificate.
L'indagine del terzo anno si è svolta in due particolari zone del Trentino definite come regioni modello. La regione di Nanno è rappresentativa della Val di Non, nelle zone di alta incidenza di infezione, l'altra si colloca a Piovi, in Val d'Adige, nelle zone di bassa incidenza.

Per verificare la presenza del fitoplasma è stata utilizzata la tecnica di PCR sul DNA dei campioni con appropriate coppie di primers.
L'analisi della variabilità genetica del fitoplasma è stata effettuata in un primo momento mediante PCR-RFLP con primers ed enzimi di restrizione idonei alla determinazione del ceppo. Successivamente è stato messo a punto un saggio di microsequenziamento mediante elettroforesi capillare (SNaPshot^{TM}) che permette analisi molto più accurate, efficienti e veloci rispetto alla tecnica di PCR-RFLP.
Un test di q-PCR con sonde TaqMan MGB è stato sviluppato per l'analisi quantitativa del patogeno. La clonazione in un plasmide di un frammento di DNA del fitoplasma è stata necessaria per la preparazione della curva standard richiesta per l'analisi.
Anche gli afidi trovati positivi al fitoplasma sono stati analizzati mediante q-PCR per determinare la quantità di fitoplasma presente. Alcune *C. picta* raccolte nella stessa area sono state analizzate insieme per avere così un riferimento su cui confrontare i dati.
Infine è stata eseguita una prima analisi quantitativa su testa e corpo di alcuni campioni di afidi e psille per vedere come il fitoplasma è distribuito nell'insetto.

Da questo lavoro è emerso che il ceppo AT2 è ampiamente diffuso in tutto il Trentino. Nelle Valli di Non e di Sole è presente quasi esclusivamente questo sottotipo. Proseguendo da nord verso sud, aumenta la percentuale del ceppo AT1. Il ceppo AP è raro e si trova esclusivamente in Val di Non. Dall'analisi dei dati è stata riscontrata un'altissima correlazione tra i ceppi e la zona geografica mentre non si sono rilevate particolari connessioni tra i ceppi del fitoplasma e le cultivar, i portainnesti e l'età della pianta.
Nelle valli a nord-ovest del Trentino la struttura della popolazione del patogeno rimane invariata nel tempo, mentre nelle altre vallate la presenza del ceppo AT1 diminuisce fino a dimezzarsi.
L'intensificarsi del campionamento, come nel caso delle micro-aree, non mostra frequenze dei sottotipi diverse.
Le psille riflettono la stessa distribuzione dei ceppi trovata nelle piante infette, all'interno di ogni zona sia nel 2002 che nel 2004. Si ha quindi conferma della correlazione tra i ceppi e l'area geografica.
Non si è trovata nessuna connessione tra i ceppi e le specie di psilla mentre non si è potuto valutare lo stadio di sviluppo per la mancanza di campioni rappresentativi.

Si dimostra chiaramente che la regione modello di Nanno è effettivamente rappresentativa della Val di Non con una netta prevalenza del ceppo AT2 e qualche caso di infezione causata dal ceppo AP. Nella regione modello di Piovi, l'assenza del ceppo AP e la presenza di AT1 rispecchiano la distribuzione presenti nelle valli al centro-sud del Trentino.

Dall'analisi quantitativa emerge il fatto che la concentrazione del ceppo AT1 è più elevata rispetto agli altri, ma il ristretto numero di campioni analizzati non permette di confermare questo dato senza ulteriori indagini. Sembra comunque che i campioni di piante con il portainnesto M9 presentino concentrazioni più elevate, come le piante con età relativamente giovane.

Dall'analisi sugli afidi risulta chiaro che sono in grado di acquisire una discreta concentrazione di fitoplasma, anche se molto più bassa rispetto a quella presente nelle psille. Nel corpo di tutti gli insetti è stata riscontrata una più alta concentrazione del patogeno rispetto alla testa corrispondente.

Considerando anche i risultati negativi di alcune prove di trasmissione svolte all'Istituto Agrario di San Michele all'Adige sembra comunque che gli afidi non abbiano un ruolo rilevante nella diffusione della malattia.

1 - INTRODUCTION

The well-known phytoplasmal diseases are over 700 so far and their economic impact is connected to spatial spread, time stability, seriousness and the kind of cultivation involved. The phytoplasma-susceptible plant species include many important food, vegetable, and fruit crops, ornamental plants, and timber and shade trees. The list of diseases caused by phytoplasmas continues to grow. Many newly emerging diseases have been identified in the last few years.

Phytoplasmal infections are the primary limiting factors for production of many important crops all over the world. Because of these diseases, the movement of many of the affected plant species are restricted by quarantine regulations internationally.

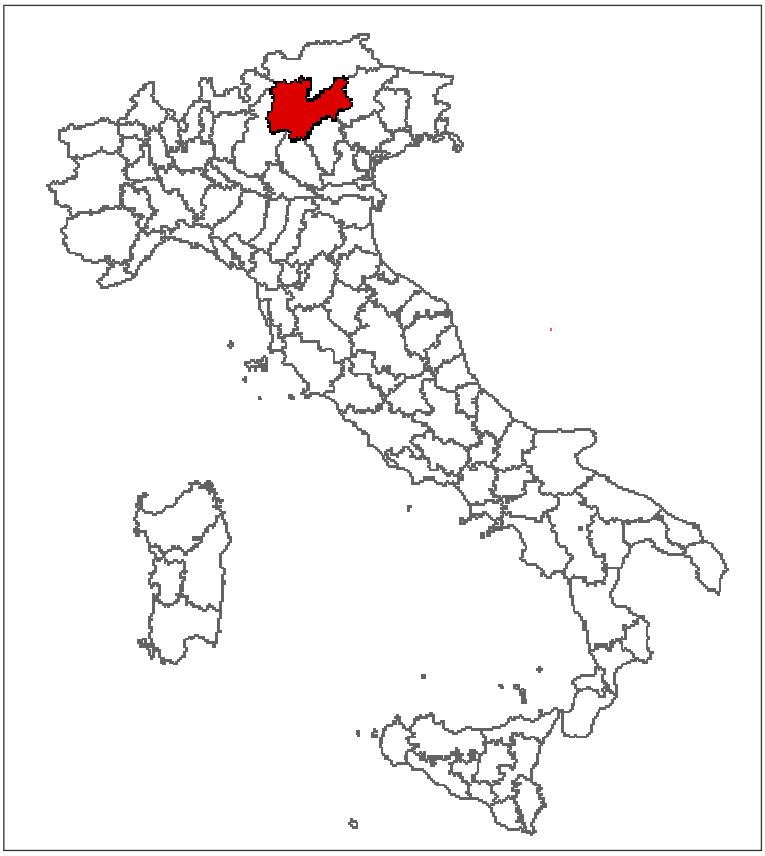
In reference to the horticulture and viticulture of North Italy, the major phytoplasmal diseases are the following: Apple Proliferation (AP), Pear Decline (PD), European Stone Fruit Yellows (ESFY), Flavescence dorée (FD) and Bois noir (BN).

Singularly in the Trentino region, (North Italy, **Fig. 1**) where the apple growing is well developed, AP is a very widespread important disease and has been known for the last fifty years (Rui, 1950).

Since 1998 the disease has spread in the zones of Val di Non and Val di Sole with considerably greater frequency. All commercially fine cultivars and apple rootstocks are susceptible to AP, especially the cv. *Golden delicious*, *Renetta del Canada* , *Gala* and the *Red delicious* cultivar group grafted on various rootstocks (Vindimian and Delaiti, 1996).

This problem is also spread in the most important European fruit growing areas.

Fig. 1: Trentino region (red colour) located in North Italy.



1.1 - PHYTOPLASMAS

1.1.1 - Introduction

In 1967, Japanese scientists discovered that plant pathogens known as phytoplasmas (previously termed mycoplasmalike organisms) were the probable cause of plant yellows diseases (Doi *et al.*, 1967). Before that discovery, all yellows diseases of plants had been presumed to be caused by viruses, although viruses could not consistently be visualized in diseased tissues or isolated from infected plants. To date, these unique plant pathogens have been associated with diseases in several hundred plant species. Phytoplasmas are minute bacteria without cell walls that inhabit phloem sieve elements (specialized cells for the translocation of nutrients in plants) in infected plants (Lee and Davis, 1992). In nature, phytoplasmal plant diseases are spread by sap-sucking insect vectors. Unlike most human and animal mycoplasmas, phytoplasmas cannot be cultured *in vitro*. Traditionally, the

identification and classification of phytoplasmas were based primarily on such biological properties as the symptoms induced in infected plants, plant host range, and relationships with insect vectors (Chiykowski, 1991; Errampalli *et al.*, 1991; Shiomi and Sugiura, 1984). The advances in molecular-based biotechnology have made it possible to gain new knowledge about phytoplasmas and to develop systems for their accurate identification and classification. Molecular-based probes, such as mono- and polyclonal antibodies, and cloned phytoplasma DNA fragments developed in the 1980s (Chen *et al.*, 1992) have been used to detect various phytoplasmas associated with plants and insects and to study their genetic interrelationships.

A major breakthrough in the understanding of phytoplasmas began in the late 1980s and early 1990s. At first, phylogenetic analysis of 16S rRNA and ribosomal protein (rp) gene sequences revealed the phylogenetic position of phytoplasmas, definitively placing them as members of the class Mollicutes (Kuske and Kirkpatrick, 1992; Lim and Sears, 1992; Seemüller *et al.*, 1994). The phylogenetic analyses formed the basis for a provisional taxonomic system for phytoplasmas. Subsequently, universal (generic) oligonucleotide primers based on conserved 16S rRNA gene sequences were designed and used in polymerase chain reaction (PCR) assays that allowed, for the first time, detection of a broad array of phytoplasmas associated with plants and insect vectors (Ahrens and Seemüller, 1992; Deng and Hiruki, 1991 I; Gundersen and Lee, 1996; Namba *et al.*, 1993 I). A comprehensive classification scheme was constructed based on restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rDNA sequences (Lee *et al.*, 1994). For the first time, the identities of numerous phytoplasmas associated with hundreds of diseases were determined unambiguously. This progress has greatly facilitated studies on both the ecology and genomic diversity of phytoplasmas and the epidemiology and physiology of phytoplasmal diseases.

1.1.2 - Morphology and structure

Yellows diseases have been known since the early 1900s. One such disease, aster yellows, was first reported in 1902 (Kunkel, 1926). Before 1967, its causal agent was thought by plant pathologists to be of viral origin because it could not be cultured in artificial media and could pass through a bacteria-proof filter. In 1967, Doi *et al.* (1967) discovered that particles in ultrathin sections of the phloem of plants affected by yellows diseases, including aster yellows, resembled animal and human mycoplasmas. The agents associated with these plant yellows diseases were pleiomorphic in shape, with a size range similar to that of mycoplasmas. They lacked rigid cell walls, were surrounded by a single unit membrane, and

were sensitive to tetracycline antibiotic (Doi *et al.*, 1967). The findings were consistent with the nature of the agents as bacteria lacking cell walls, and led to a drastic change in perception of the etiology of many yellows diseases.

Although phytoplasmas, in single cross sections, appear as rounded pleiomorphic bodies with an average diameter ranging from 200 to 800 µm, other studies revealed a filamentous morphology. For example, with serial sections of sieve elements from phytoplasma-infected tissues,Waters and Osborne (1978) demonstrated that many phytoplasmas were filamentous. Haggis and Sinha (1978) also observed short, branched, filamentous forms in sieve elements with clover phyllody and aster yellows phytoplasmas using scanning electron microscopy. Using dark-field microscopy, Lee and Davis (1983) observed that filamentous bodies were predominant in isolated transparent sieve elements prepared by enzymatic digestion of veinal tissues infected with several phytoplasmas. Filamentous bodies were especially predominant in infected plant tissues during the early stages of infection.

1.1.3 - Symptoms in phytoplasma infected plants

Plants infected by phytoplasmas exhibit an array of symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators (Chang, 1998; Chang and Lee, 1995). Symptoms include virescence (the development of green flowers and the loss of normal flower pigments), phyllody (the development of floral parts into leafy structures), sterility of flowers, proliferation of auxiliary or axillary shoots resulting in a witches' broom appearance, abnormal elongations of internodes resulting in slender shoots, generalized stunting (small flowers and leaves and shortened internodes), discolorations of leaves or shoots, leaf curling or cupping, bunchy appearance of growth at the ends of the stems, and generalized decline (stunting, die back of twigs, and unseasonal yellowing or reddening of the leaves). The symptoms induced in diseased plants vary with the phytoplasma and with the stage of infection. Internally, phytoplasmal infections can cause extensive phloem necrosis and, often, excess formation of phloem tissue, resulting in swollen veins. In general, symptoms induced by phytoplasmal infection have a clearly detrimental effect on plants, although some plant species are tolerant or resistant to phytoplasmal infections. Such plants may be symptomless or exhibit mild symptoms. Economic losses caused by phytoplasmal infections range from partial reduction in yield and quality to nearly total crop loss. In one rare case, phytoplasmal infection of poinsettias produces symptoms that are beneficial to growers. The bushy growth form and dwarfing of poinsettias resulting from

phytoplasmal infection is a desirable trait that is essential for the production of showy multiflowered potted poinsettia plants for holiday celebrations.

1.1.4 - Transmission and spread of phytoplasmal diseases

Phytoplasmas are phloem-limited plant pathogens that are found primarily in the sieve elements of infected plants. Phytoplasmal diseases are spread primarily by sap-sucking insect vectors belonging to the families Cicadellidea (leafhoppers) and Fulgoridea (planthoppers) (Tsai, 1979). Insects feed on phloem tissues, where they acquire phytoplasmas and transmit them from plant to plant. Phytoplasmas may overwinter in infected vectors, as well as in perennial plants that serve as reservoirs of phytoplasmas that are spread in the following spring.

Thus far, there has been no substantial evidence to indicate that phytoplasmal diseases are seed-borne. However, phytoplasmas can be spread by vegetative propagation through cuttings, storage tubers, rhizomes, or bulbs. Phytoplasmas that cause many ornamental and fruit tree diseases apparently are spread by vegetative propagation. Phytoplasmas can be transmitted through grafts; they cannot, however, be transmitted mechanically by inoculation with phytoplasma-containing sap.

1.1.5 - Detection, identification, and classification of phytoplasmas

1.1.5.1 - Serological and DNA-DNA Hybridization Assays

In past decades, because of the inability to obtain pure cultures of any phytoplasma, their detection and identification were never precise. The presence of characteristic symptoms in diseased plants and subsequent observation of mycoplasmalike bodies in ultrathin sections of diseased plants were the main criteria used to diagnose diseases of possible phytoplasmal origin (Chen and Chen, 1998; Chiykowski *et al.*, 1991; Cousin *et al.*, 1986; Doi *et al.*, 1967; Haggis and Sinha, 1978). In some cases, the disappearance of symptoms after antibiotic (i.e. tetracycline) treatment provided additional evidence to support the diagnosis (Doi *et al.*, 1967). Phytoplasma strains were differentiated and identified by their biological properties, such as the similarity and difference in symptoms they induced in infected plants, their plant hosts, and their insect vectors (Chiykowski and Sinha, 1989; Kunkel, 1926).

In the 1980s, the development of molecular probes such as poly- and monoclonal antibodies, and cloned phytoplasma-specific DNA, advanced the art of phytoplasmal disease diagnostics

(Chen *et al*., 1992; Davis *et al*., 1991; Firrao *et al*., 1993; Kirkpatrick *et al*., 1987; Sears *et al*., 1989). Serological tests, such as enzyme-linked immunosorbent assay and immunofluorescence microscopy, using highly specific monoclonal antibodies, provided relatively simple, sensitive, and reliable means for the detection and identification of specific phytoplasma strains (Chen *et al*., 1992). Dot and Southern hybridizations using cloned phytoplasma DNA probes permitted studies of genetic interrelationships among phytoplasmas, resulting in the recognition of several distinct phytoplasma groups (genomic strain clusters) and subgroups (subclusters) (Griffiths *et al*., 1994; Kison *et al*., 1994; Ko *et al*., 1994). Differentiation of strains within a given strain cluster was easily achieved by Southern hybridization and RFLP analysis of phytoplasma genomic DNA. Lee *et al.* (1992) used a substantial number of cloned phytoplasma DNA probes in RFLP analyses to establish the first genotype-based classification scheme for differentiation of strains in the aster yellows phytoplasma genomic cluster. Their results revealed that closely related phytoplasma strains can cause distinct kinds of symptoms in infected plants and that two distinct strains may induce similar symptoms, underscoring the inaccuracy resulting from the previous use of symptoms as parameters for differentiating phytoplasma strains. Currently, the highly reliable molecular-based detection and classification systems have largely replaced the traditional biologically oriented classification systems.

1.1.5.2 - Polymerase Chain Reaction

PCR-based assays developed in the late 1980s and early 1990s further advanced diagnostics for phytoplasmal diseases. PCR assays provide a much more sensitive means than serological tests or DNA-DNA hybridization assays for detection of phytoplasmas. Initially, PCR primers were designed based on sequences of cloned phytoplasma DNA fragments and were used to detect specific phytoplasmas (Deng and Hiruki, 1991 II; Goodwin *et al.*, 1994; Harrison *et al.*, 1994; Jarausch *et al.*, 1994; Schaff *et al.*, 1992). PCR using these primers facilitated detection of low titers of phytoplasmas that were not readily detected by serological or DNA-DNA hybridization assays. Subsequently, several research groups during the late 1980s and early 1990s designed phytoplasma universal (generic) or phytoplasma group-specific oligonucleotide primers that were based on highly conserved 16S rRNA gene sequences (Ahrens and Seemüller, 1992; Davis and Lee, 1993; Deng and Hiruki, 1991 I). This progress enabled amplification of 16S rDNA sequences from a broad spectrum of phytoplasma strains and from specific strains belonging to a given phytoplasma group. This accomplishment was a major breakthrough in the field of phytoplasma research. For the first time, it was feasible

for researchers to detect and study the whole spectrum of phytoplasma strains associated with plants or insect vectors worldwide. Universal and phytoplasma group-specific primers were also developed based on the 16S-23S intergenic spacer region sequences or conserved rp gene and elongation factor EF-Tu (*tuf*) gene sequences (Firrao *et al.*, 1993; Gundersen *et al.*, 1996; Lim and Sears, 1991; Lorenz *et al.*, 1995; Schneider *et al.*, 1995; Smart *et al.*, 1996).

1.1.5.3 - RFLP analysis

For decades, the lack of a comprehensive classification scheme for phytoplasmas hindered phytoplasmal research in almost every aspect. Etiologies of numerous puzzling diseases and the identities of the phytoplasmas associated with such diseases could not be clarified. The use of PCR to amplify 16S rRNA gene sequences made it possible to detect a wide array of phytoplasmas associated with diseases in hundreds of plant species. RFLP analysis of PCR-amplified 16S rDNA sequences with a number of restriction enzymes was used by Schneider *et al.* (1993) to differentiate various phytoplasmas by their distinct RFLP patterns. This procedure proved to be simple, reliable, and practical. Based on RFLP analyses (with 15–18 restriction enzymes) of 16S rDNA amplified from representative phytoplasma strains associated with numerous diseases, Lee *et al.* (1993) proposed a classification scheme that comprised 10 major phytoplasma groups (termed 16S rRNA groups) and 15 subgroups. The scheme was later expanded to14 groups and 38 subgroups. The phytoplasma 16S rRNA groups identified by RFLP analyses are consistent with phytoplasma subclades delineated based on phylogenetic studies (Gundersen *et al.*, 1994). This comprehensive classification scheme, combined with illustrative RFLP patterns characteristic of each distinct group and subgroup, continues to provide a simple, reliable, and practical means to identify unknown phytoplasmas without the need to sequence the 16S rRNA gene.

1.1.6 - Taxonomy and Phylogenesis

1.1.6.1 - Phytoplasma taxonomy

If till 1967 the causal agent of yellows diseases seemed to be of viral origin, from 1967 it was associated to mycoplasma. From 1967 to 1994, the term mycoplasmalike organisms or MLOs was used to refer to the presumed causal agents of many yellows diseases (McCoy *et al.*, 1989).

The taxonomy of the unculturable phytoplasmas is a particular problem because many criteria important in the taxonomy of culturable bacteria are only attainable with difficulty or not at all. This applies mainly to suitable phenotypic information. Thus, the criteria of modern bacterial taxonomy as a polyphasic approach to classification, in which genotypic, phylogenetic, and phenotypic information is integrated (Stackebrandt and Goebel, 1994), cannot be employed in phytoplasmology. As similar problems exist with other nonculturable prokaryotes, a formal classification of such bacteria, which are extensively characterized by molecular techniques, has been proposed, using a "*Candidatus*" prefix (Murray and Schleifer, 1994).

The Working Team on Phytoplasmas of the International Research Programme of Comparative Mycoplasmology (IRPCM) of the International Organization for Mycoplasmology (IOM) has, at its meetings in 1994 and 1996 at Bordeaux (France) and Orlando (Florida), proposed to adopt this new classification based on 16S rRNA gene sequences and describe each subclade (major group) defined to the tentative species level as *Candidatus* Phytoplasma species (Seemüller *et al.*, 1998).

Particularly sequence analysis of 16S rDNA, have revealed that phytoplasmas constitute a coherent, genus-level taxon. Also, several provisional species have been described to date and rules for future putative species delineation have been defined (IRPCM Phytoplasma/ Spiroplasma Working Team – Phytoplasma taxonomy group, 2004). According to these recommendations, "a phytoplasma can be described as a novel *Candidatus* Phytoplasma species if its 16S rDNA sequence has <97,5% similarity to that of any previously described *Candidatus* Phytoplasma species". In cases where phytoplasmas share ≥97,5% 16S rDNA sequence similarity, description as different *Candidatus* species is only recommended if there is an indication that these phytoplasmas clearly represent separate populations, as evidenced by significant differences based on molecular markers other than 16S rDNA, antibody specificity, host range and vector transmission specificity.

The ICSB Subcommittee on the Taxonomy of *Mollicutes* (1993, 1997) has agreed to this proposal and adopted the policy of basing phytoplasma taxonomy on phylogeny.

Till 2000 five *Candidatus* phytoplasmas species have been proposed. Again, in 2004 about Apple Proliferation (or 16SrX) group, Seemüller and Schneider (2004) proposed that the AP, PD and ESFY phytoplasmas were designated as novel, distinct *Candidatus* species: "*Candidatus* Phytoplasma mali", "*Candidatus* Phytoplasma pyri" and "*Candidatus* Phytoplasma prunorum".

It was possible to divide the phytoplasmas examined into about 20 major groups (**Tab. 1**), in which a total of 75 phytoplasmas could be distinguished. The major groups are distinctly different and are planned, according to the consensus reached, to be delineated to the species level. Such a formal description as *Candidatus* species will undoubtedly improve the current situation, complicated by confusing historical and preliminary classification schemes.

Tab. 1: classification of phytoplasmas based on restriction fragment length polymorphism analysis of 16S rRNA and ribosomal protein sequences.

GROUP	SUBGROUP	MAJOR DISEASE
16SrI		**Aster Yellows**
	16SrI-A	Aster Yellows
	16SrI-B	Maryland Aster Yellows
	16SrI-C	Clover Phyllody
	16SrI-D	Paulownia Witches' Broom
	16SrI-E	Blueberry Stunt
	16SrI-F	Apricot Chlorotic Leaf Roll
	16SrI-K	Strawberry Multiplier
16SrII		**Peanut Witches' Broom**
	16SrII-A	Peanut Witches' Broom
	16SrII-B [1]	Lime Witches' Broom
	16SrII-C	Faba Bean Phyllody
	16SrII-D	Sweet Potato Little Leaf
	16SrII-E [2]	Papaya Yellow Crinkle
16SrIII		**X-disease**
	16SrIII-A	Peach X-disease
	16SrIII-B	Clover Yellow Edge
	16SrIII-C	Pecan Bunch
	16SrIII-D	Golden Rod Yellows
	16SrIII-E	Spirea Stunt
	16SrIII-F	Milkweed Yellows
	16SrIII-G	Walnut Witches' Broom
	16SrIII-H	Poinsettia Branching Inducing
16SrIV		**Coconut Lethal Yellows**
	16SrIV-A	Coconut Lethal Yellows
	16SrIV-B	Tanzanian Lethal Decline
16SrV		**Elm Yellows**
	16SrV-A	Elm Witches' Broom
	16SrV-B	Cherry Lethal Yellows
	16SrV-C	Flavescence Dorèe
16SrVI		**Clover Proliferation**
	16SrVI-A	Clover Proliferation
	16SrVI-B	Strawberry Multiplier
	Unclassified	Willow Witches' Broom
16SrVII	[3]	**Ash Yellows**
16SrVIII		**Loofah Witches' Broom**
16SrIX		**Pigeon Pea Witches' Broom**
	16SrIX-A	Pigeon Pea Witches' Broom
	Unclassified	Echium Vulgare Yellows
16SrX		**Apple Proliferation**
	16SrX-A [4]	Apple Proliferation
	16SrX-B [5]	European Stone Fruit Yellows
	16SrX-C [6]	Pear Decline
16SrXI		**Rice Yellow Dwarf**

16SrXI	16SrXI-A	Rice Yellow Dwarf
	16SrXI-B	Sugarcane White Leaf
	Unclassified	Leafhopper Borne
16SrXII		**Stolbur**
	16SrXII-A	Stolbur
	16SrXII-B [7]	Australian grapevine yellows
	[8]	Japanese Hydrangea Phyllody
16SrXIII		**Mexican Periwinkle Virescence**
	16SrXIII-A	Mexican Periwinkle Virescence
	16SrXIII-B	Strawberry Green Petal
16srXIV		**Bermuda Grass White Leaf**
Undesignated		Italian Bindweed Stolbur
Undesignated		Buckthorn Witches' Broom
Undesignated		Spartium Witches' Broom
Undesignated		Italian Alfalfa Witches' Broom
Undesignated		Cirsium Phyllody

[1] *Candidatus* Phytoplasma aurantifolia

[2] *Candidatus* Phytoplasma australasia

[3] *Candidatus* Phytoplasma fraxini

[4] *Candidatus* Phytoplasma mali

[5] *Candidatus* Phytoplasma prunorum

[6] *Candidatus* Phytoplasma pyri

[7] *Candidatus* Phytoplasma australiense

[8] *Candidatus* Phytoplasma japonicum

1.1.6.2 - Phylogenetic position of phytoplasmas

In the 1980s, Woese *et al.* (1980) and others developed an innovative procedure that permitted studies of the phylogeny of prokaryotes by analyses of highly conserved rRNA gene sequences. Woese (1987) suggested that the heterogeneous mollicutes were derived from a single lineage of ancestral grampositive bacteria. Comparison of 16S rRNAgene sequences among members of the Mollicutes class and several walled prokaryotes by Weisburg *et al.* (1989) indicated that the mollicutes arose from a gram-positive clostridiumlike bacterial ancestor of the lactobacillus lineage, whose genome has low guanine plus cytosine (G+C) content. Four major phylogenetic groups (clades) were identified: *Mycoplasma hominis, M. pneumoniae, Spiroplasma*, and *Anaeroplasma* clades. The *Anaeroplasma* clade consisted of two orders: Anaeroplasmatales and Acholeplasmatales. Phylogenetic investigations of MLOs began in the late 1980s Lim and Sears (1989) compared 16S rRNA gene sequences from an MLO (*Oenothera* virescence phytoplasma) belonging to the aster yellows group with *Acholeplasma laidlawii* and an animal mycoplasma; their

findings revealed that the phytopathogenic MLO was a member of class Mollicutes and that the MLO was more closely related to *Acholeplasma* than to the animal mycoplasma. Subsequent studies analyzing sequences from several ribosomal proteins confirmed the close phylogenetic relationship between this MLO and *Acholeplasma* (Sears and Kirkpatrick, 1994; Toth *et al.*, 1994). These discoveries inspired further investigations on the phylogenetic relationships of various MLOs by several other research groups (Namba. *et al.*, 1993 II).

Global phylogenetic analyses of 16S rRNA and rp gene operon sequences showed that phytoplasmas formed a large discrete monophyletic clade within the expanded *Anaeroplasma* clade (Gundersen *et al.*, 1994). The phytoplasma clade is paraphyletic to *Acholeplasma* species (**Tab. 2**). *Acholeplasma palmae* and *A. modicum* are the closest known relatives of phytoplasmas.

Tab. 2: taxonomy of phytoplasma.

Superkingdom	Bacteria
Phylum	Firmicutes
Class	Mollicutes
Order	Acholeplasmatales
Family	Acholeplasmataceae
Genus	Phytoplasma

Within the phytoplasma clade, about 20 subclades have been recognized (Seemüller *et al.*, 1998). These comprehensive phylogenies have formed a basis for classification of this uncultured plant pathogen group. The phylogenetic subclades coincide with16S rRNA phytoplasma groups identified by their RFLP patterns, validating the classification schemes that are based on RFLP analysis of 16S rDNA sequences (**Tab. 1**).

Additional phylogenetic markers such as rp genes, the *tuf* gene, 23S rRNAgene, and the 16S/23S rRNA intergenic spacer sequences have been used as supplemental tools for differentiation of phytoplasmas (Kirkpatrick *et al.*, 1994; Smart *et al.*, 1996). Phytoplasma group delineation using these markers has been consistent with that deduced by analysis of the16S rRNA gene. However, the number of subgroups resolved by each marker is slightly different.

1.1.7 - Phytoplasma genome

1.1.7.1 - Base composition of genome

Because phytoplasmas cannot be cultured *in vitro*, the genomic properties of uncultured phytoplasmas have been determined using partially purified phytoplasmas or phytoplasma-enriched preparations from infected plants or insect vectors. Using chromosomal DNA linearized by gamma irradiation and pulsed-field gel electrophoresis for DNA separation, Neimark and Kirkpatrick (1993) and Marcone *et al.* (1999) reported that the sizes of phytoplasma genomes vary considerably, ranging from 530 to 1350 kilobase pairs (kbp). The Bermuda grass white leaf phytoplasma represents the smallest genome size (530 kbp) found in phytoplasmas to date and may represent the smallest chromosome known for any living cell (Mushegian and Koonin, 1996). The genome sizes of phytoplasmas are similar to those of members of the genus *Mycoplasma* (580–1300 kbp), order *Mycoplasmatales*, but are smaller than their closest relatives, members of the genus *Acholeplasma* (~1600 kbp), order *Acholeplasmatales* (Razin *et al.*, 1998). Similar to other members of the *Mollicutes*, phytoplasmas contain one circular double-stranded chromosomal DNA molecule (Niemark and Kirkpatrick, 1993). However, in DNA preparations from plants infected by some aster yellows phytoplasma strains, two separate chromosomes were detected (Niemark and Kirkpatrick, 1993). Whether the presence of two chromosomes was caused by infection by two phytoplasmas in the same plant has not been clarified. Short circular extrachromosomal DNAs (1,7–7,4 kbp) or plasmids were found in all members of the aster yellows group (16SrI) and stolbur group (16SrXII) and in some members of the X-disease (16SrIII) and clover proliferation (16SrVI) groups. Large plasmids may also be present in some phytoplasmas. Some small plasmids may be of viral origin. Two extrachromosomal DNAs have been sequenced and shown to share significant sequence similarity with genes in geminiviruses, a type of DNA plant virus (Kuboyama *et al.*, 1998). The GCC contents of phytoplasma chromosomal DNA are estimated to be between 23 and 29 mol % based on estimates from buoyant density centrifugation (Kollar and Seemüller, 1989). The low GCC contents of phytoplasma DNA support the phylogenetic affiliation of phytoplasmas with members of class *Mollicutes* (Razin *et al.*, 1998).

1.1.7.2 - Genes

Genetic information about uncultured phytoplasmas is scarce. To date, only a few genes, 16S rRNA, 23S rRNA, rp operon, *tuf* gene, and two genes encoding membrane proteins have been characterized (Berg *et al.*, 1998; Berg and Seemüller, 1998; Chen *et al.*, 1994; Guo *et al.*, 1998; Yu *et al.*, 1998). Two extrachromosomal DNAs of onion yellows phytoplasma have been sequenced and shown to share significant sequence similarity with genes in germini viruses, a type of plant DNA virus (Kuboyama *et al.*, 1998). The extrachromosomal DNA of sugarcane white leaf phytoplasma has also been sequenced (Nakashima *et al.*, 1993). Of these genes, the16S rRNA gene is the best characterized. Currently, 16S rRNA genes from >60 distinct phytoplasmas have been sequenced (Seemüller *et al.*, 1998). Phytoplasmas share from 88% to >99% sequence similarity among themselves and share 87%–88,5% similarity with their closest relatives, *Acholeplasma* spp. (Gundersen *et al.*, 1994). Certain 16S rRNA oligonucleotide sequences (signatures) unique to phytoplasmas distinguish them from *Acholeplasma* and other members of the class *Mollicutes*. PCR primers designed on the basis of these unique sequences have been widely used for specific detection of phytoplasmas in infected plant tissues and insect vectors. Also 23S rRNA genes from several strains of X-disease phytoplasma group were sequenced and characterized. These 23S rRNA gene sequences were found to be equally or more highly conserved than 16S rRNA sequences among members of the X-disease phytoplasma group (Guo *et al.*, 1998).

The rRNA genes in phytoplasmas are organized in the same order as in other eubacteria: 50 16S rRNA-spacer region-23S rRNA 30. Sequence analysis of the spacer region revealed that a single tRNAile (isoleucine transfer RNA) is present in all phytoplasmas (Kirkpatrick *et al.*, 1990; Kuske and Kirkpatrick, 1992). This is different from animal mycoplasmas, in which the tRNA gene is absent in the spacer region. Hybridization analyses using a 16S rRNA gene probe indicated the presence of two sets of 16S rRNA operons (Smart *et al.*, 1996). Heterogeneity of the two operons is apparent in some phytoplasmas (Davis *et al.*, 1998; Liefting *et al.*, 1996).

A segment of an rp operon (*rps3* and *rpl22*) of an aster yellows phytoplasma strain was cloned and sequenced by Lim and Sears (1991) and Toth *et al.* (1994). The deduced amino sequence data from these two genes revealed that this phytoplasma is more closely related to acholeplasmas than to other members of the *Mollicutes* class, which is consistent with the phylogenetic relationship that is based on analysis of 16S rRNA gene sequences. The closer relationship between the phytoplasma and acholeplasmas was further supported by the finding that both phytoplasma and acholeplasma do not use the UGA as a tryptophan codon, in

contrast to its usage in animal mycoplasmas. The rp gene sequences reveal more variations than do 16S rRNA gene sequences among phytoplasmas. In a comparison with *rpl22* gene sequences from 11 phytoplasma strains representing 8 major groups and 2 *Acholeplasma* spp., Gundersen *et al.* (1994) showed that the phytoplasmas share 60%–79% rp sequence similarities among themselves and share 50%–57% similarities with the *Acholeplasma* spp.

Phytoplasmas contain one *tuf* gene operon (Berg and Seemüller, 1998). Sequence analyses of *tuf* genes from various phytoplasma groups indicated that the *tuf* gene is at least as conserved as 16S rRNA gene sequences in phytoplasmas. Within the same phytoplasma group, there is from 96% to >99% *tuf* gene sequence similarity among members, whereas there is >90% similarity among members of different groups (Schneider *et al.*, 1997).

1.1.8 - Ecology of phytoplasmas

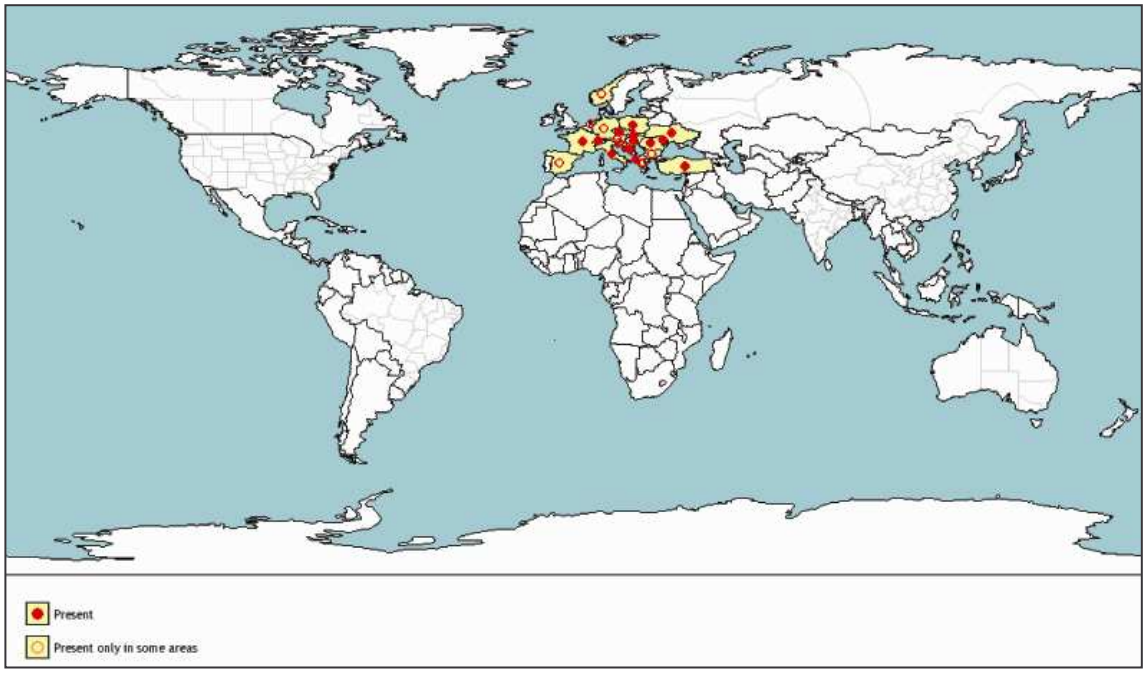
1.1.8.1 - Distribution of phytoplasmas

Phytoplasmas have been associated with diseases in several hundred plant species belonging to 98 families and with numerous homopterous insect vectors, primarily belonging to the family Cicadellidea (leafhoppers). Geographically, the occurrence of phytoplasmas is worldwide. They have been reported in at least 85 nations (McCoy *et al.*, 1989).

The development of phytoplasma-specific molecular probes, sensitive PCR assays, and comprehensive classification schemes in the last decade has greatly advanced the diagnostics of diseases caused by phytoplasmas. For the first time, the identities of phytoplasmas associated with a wide array of insect vectors and plant diseases that have been reported in the past can now be accurately determined. In the last years, numerous diseases of previously unknown etiologies were found to be caused by phytoplasmas. Evidently, similar symptoms can be induced by different types of phytoplasmas, whereas different types of symptoms can be induced by closely related phytoplasmas (Martini *et al.*, 1998). Some results have revealed that phytoplasmas are more diverse than previously thought and that they are not distributed uniformly over all continents. Many seem to be restricted to one continent or to a specific geographical region. For example, the ash yellows group (16SrVII), the clover proliferation group (16SrVI), and most of the X-disease group (16SrIII) of phytoplasmas appear to be restricted to the American continent or western hemisphere, whereas the peanut witches' broom group (16SrII) and rice yellow dwarf group (16SrXI) of phytoplasmas are restricted to the Southeast Asian region, and the apple proliferation group (16SrX) and stolbur subgroup

(16SrXII-A) are restricted to the European continent (**Fig. 2**). Geographical isolation of some phytoplasmas seems to be correlated with the distribution of their host plants and the insect vectors that are native in the particular region. For instance, maize bushy stunt phytoplasma [16SrI-B(rp-L)] is restricted to Central and South America and part of North America. These regions correspond to the geographical range of the insect vectors *Dalbulus madis* and *D. elimatus* (Davis *et al.*, 1988; Harrison, 1996).

Fig 2: distribution map of *Ca*. Phytoplasma mali belonging to Apple Proliferation group (www.eppo.org/QUARANTINE/listA2.htm).



The uniqueness of the vegetation and insect species on a given continent or in a particular geographical region, however, tends to diminish as transcontinental or interregional activities increase. Micro- and macroecosystems in each continent can change owing to a lack of conservation or through the introduction of foreign germplasms (e.g. weeds and cultivated crops) and/or insects. Thus, the phytoplasma associated with an original plant host can become dispersed and redistributed throughout geographical regions or continents. Many phytoplasmas apparently have spread well beyond the regions where they originated, especially if similar vegetation and insect vectors existed in the new ecological niches. Some phytoplasmas (e.g. aster yellows phytoplasma subgroup 16SrI-B) have become dispersed

worldwide, whereas others have become isolated in new ecological niches and have evolved independently from parental strains.

1.1.8.2 - Host specificity of phytoplasmas

The natural host ranges of phytoplasmas in insect vectors and plants vary with the phytoplasma strain (Tsai, 1979). Experimentally, some phytoplasmas can be transmitted by polyphagous vector(s) to a wide range of host plants. However, it appears that the range of plant species that can be infected by a given phytoplasma in nature is determined largely by the number of insect vector species that are capable of transmitting the phytoplasma and by the feeding behaviors (monophagous, oligophagous, and polyphagous) of these vectors.

1.2 - APPLE PROLIFERATION

1.2.1 - The phytoplasma

Apple Proliferation (AP) is a very widespread important phytoplasmal disease due to *Ca.* Phytoplasma mali (ex AP phytoplasma). This has a close phylogenetic relationship with *Candidatus* Phytoplasma pyri and *Candidatus* Phytoplasma prunorum, respectively ex PD (Pear Decline) and ESFY (European Stone Fruit Yellows) phytoplasma. All of them are classified in the same AP cluster, the 16SrX group (Seemüller *et al.*, 1998).

The pathogen is located in sieve tubes, mainly near the sieve plates. Colonized sieve tubes can necrose during the summer. Then, replacement phloem forms containing small irregular sieve tubes which become colonized. As the phytoplasma occurs only in functional sieve tubes, its distribution in stems and shoots of infected apple trees fluctuates considerably during the year (Schaper and Seemüller, 1982; Seemüller *et al.*, 1984 II). The phytoplasma occur most frequently in stems and shoots in late summer and autumn. When the sieve tubes degenerate in winter, the pathogen form stringlike structures that eventually disappear from the aerial parts of the tree.

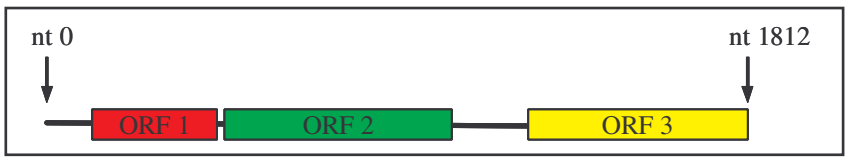
In February or March the phytoplasma cannot be found in shoots of diseased apple trees except for a few witches' brooms in which some sieve elements have remained functional. The phytoplasma passes winter in roots where functional sieve tubes are present in large numbers throughout the year. When new aerial sieve elements are formed in April or May, recolonization of the stems and branches occurs. In the period from 10 to 30 days following

full bloom, the pathogen normally reaches a height of about 120 cm in trees above the ground (Schaper and Seemüller, 1984). Although phytoplasmas are consistently in the roots, recolonization of the aerial parts does not occur every year. In older trees the shoots are often free of pathogen. In that case no witches' brooms or red leaves occur on the tree (Seemüller *et al.*, 1984 I). The distribution of phytoplasma in diseased trees was proven by fluorescence microscopy of the phloem cells and by transmission experiments (Seemüller *et al.*, 1984 II).

1.2.2 - Strain

In 2000 Jarausch *et al*. characterized three different strains of *Ca.* Phytoplasma mali by PCR-RFLP analysis of a 1812 bp non-ribosomal fragment of pathogen DNA (Jarausch *et al*., 2000). Nearly 90% of the 1812 bp nucleotide sequence was determined on both strands (Jarausch *et al*., 1994). **Figure 3** shows a schematic representation of the sequenced region. Three putative open reading frames (ORFs), each preceded by a putative Shine-Dalgarno ribosome binding sequence, could be localized.

Fig. 3: schematic representation of the 1,8-kb chromosomal fragment of *Ca.* Phytoplasma mali isolate.



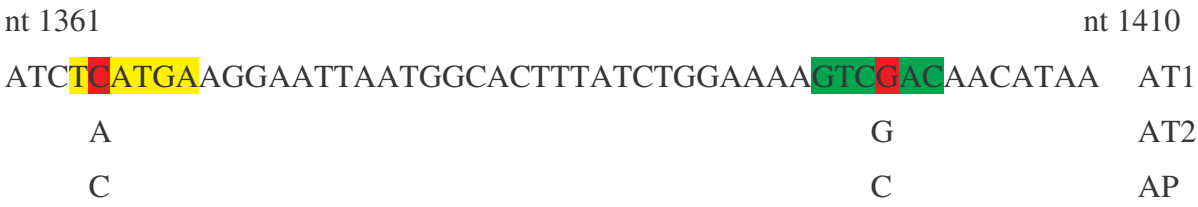
Homology searches in protein data banks revealed a significant similarity of ORF 2 with nitroreductases from *Enterobacter cloacae* (Bryant *et al.*, 1991) and *Salmonella typhimurium* (Watanabe *et al.*, 1990). ORF 3, which is incomplete, shows significant similarity to a hypothetical 40-kDa protein of *Escherichia coli* (Karow and Georgopoulos, 1991). ORF 1, for which no significant homologies could be found, is separated from ORF 2 by only 13 bp without a terminatorlike structure, suggesting a putative two-gene transcription unit. A possible terminator sequence is located in the 3'-noncoding region of ORF 2. Concerning the codon usage, all three tryptophan codons found in ORFs 2 and 3 are UGG codons and all putative stop codons are UAA codons. This is in accordance with previous results for ribosomal protein genes of a plant-pathogenic phytoplasma (Lim and Sears, 1991; Lim and

Sears, 1992) showing that in contrast to mycoplasmas, spiroplasmas, and ureaplasmas, the UGA codon does not serve as a tryptophan codon and is not used as a frequent stop codon. This supports results of 16S rDNA sequence analysis (Kuske and Kirkpatrick, 1992; Lim and Sears, 1989) that phytoplasmas are phylogenetically related to acholeplasmas. The chromosomal DNA fragment of *Ca.* Phytoplasma mali exhibits a G+C content of 19% in the coding regions and of only 7% in the noncoding regions. This finding is consistent with the A+T-rich genomes of phytoplasmas (Kollar and Seemüller, 1989).

A polymorphism profiles were observed with restriction enzymes BspH I (or Rca I) and Hinc II. From the results, three RFLP groups could be distinguished (**Fig. 4**).

Isolates of strain AT1 possess both of the restriction sites while strain AT2 is characterized by the absence of the BspH I-site at nt 1364 of the sequence. The third subtype, AP, possess the BspH I-site at nt 1364 but does not possess a Hinc II-site at nt 1400 (Jarausch *et al*., 1994).

Fig. 4: nucleotide sequence of a part of the coding region of ORF 3 on the 1,8 kbp chromosomal fragment of *Ca.* Phytoplasma mali exhibits point mutations between the three strain AT1, AT2 and AP (BspH I-site is highlighted in yellow; Hinc II-site is highlighted in green; Point mutations are highlighted in red).



Sequence analysis of the intergenic region and the truncated ORF 3 showed that only two point mutations were responsible for the observed polymorfphism.

Subtype AT2 isolates have a C to A transition at nt 1365 leading to a His instead of an Asn amino acid. Subtype AP isolates show a G to C transition at nt 1401 resulting in an Asp to a His amino acid change in ORF 3. Database comparisons revealed that the truncated aminoacid sequence of ORF 3 is highly similar to the gene product of "ybfQ" with an unknown function of *Bacillus subtilis* (47% identity and 72% similarity). The two amino acid changes observed among the *Ca.* Phytoplasma mali subtypes are both located at non-conserved positions in a homologous sequence motif shared by the hypothetical proteins from the *Ca.* Phytoplasma mali, *B. subtilis* and *Lactococcus lactis*.

Interestingly, both mutations were neither located in the intergenic region nor silent mutations in the coding region. They both resulted in amino acid changes in the putative protein "ORF 3" and altered the electric charge of the protein (Jarausch *et al*., 1994).

1.2.3 - Symptoms

The disease plants first show symptoms after 1 or 2 years from infection time. The signs can related to the whole plant or only to some branches.

The main symptom of the disease is the witches' broom pertaining to the apical portion of the plant that becomes light green.

The past few summers axillary buds on the upper part of some shoots grew prematurely because of hormonal imbalance. The lack of apical dominance in affected shoots causes the witches' brooms. The secondary shoots form an angle with the main shoot of less than 45° (**Fig. 5**).

Fig: 5: disease sample showing the narrow angle of lateral shoots.

This characteristic distinguishes shoots on infected trees from those developing from lateral buds that normally occur near the base of vigorous shoots on healthy trees. Sometimes enlarged stipules develop on the apex leaves of shoots exhibiting early stages of witches' brooms. Depending on cultivar, witches' brooms may be prevalent at the apex of the main branches or near the crown of the tree.

The fruits of infected trees are smaller than normal and have longer peduncles (**Fig. 6**). In the acute stage of the disease the medium weight of the fruit is reduced by 30 to 60%, and often the taste is bitter (reduction of carbohydrate) and herbaceous. For this reason, the fruits are not marketable and the production reduces drastically.

Fig. 6: disease apples compared with two healthy fruits (on the right).

An early, red, autumn coloration of leaves occurs on diseased trees of some cultivars, and by the end of the growing season, infected trees may show late shoot growth. The terminal bud is not closed and dormant, but develops a rosette of light green leaves with enlarged stipules (**Fig. 7**). The same symptoms also occur on the brooms (Kunze, 1989).

Fig. 7: light green leaves forming a rosette.

Leaves of trees with proliferation symptoms are more susceptible to the powdery mildew fungus than those of healthy trees (Maszkiewicz *et al.*, 1979). During summer and autumn, infections with powdery mildew are favored by the development of susceptible young leaves on the brooms and on late growth of other terminal buds.

Bud break occurs earlier in the spring on diseased trees, but blossoming is delayed slightly. The stipules are greatly enlarged, dented or notched on the first leaves and appear similar to a true leaf (**Fig. 8**). The enlarged stipules at the base of shoots are characteristic of the disease. Normally during spring, healthy trees of culinary apple cultivars develop leaves with small and narrow stipules. Broader stipules may also appear on leaves of healthy trees, but not before summer. Sometimes these may be confused with the enlarged stipules at the apex of shoots in the early stage of witches' brooms.

Some alterations occur in the roots. Small roots are reduced in length, gnarled and very crooked. When the trees are severely infected, the finer roots become entangled into dense tufts, while the larger ones do not elongate. Subterraneous witches' brooms may also arise from large roots near the trunk (Kunze, 1989).

Fig. 8: stipules greatly enlarged in a disease leaf sample.

The deformations of the smaller roots prevent adequate tree nutrition (Schmidle and Kunze, 1972). In fruit bearing trees the nitrogen content of the leaves is reduced by 8 to 12%. In experimentally infected young trees, the mineral content or leaves can be lowered by as much as 23% for nitrogen, 36% for potassium and 43% for calcium compared with readings far healthy trees. The effects of these nutrition deficiencies include lighter leaf colour and reduced growth.

Only witches' brooms, including their early stage, and the enlarged stipules on basal leaves, are distinguishing symptoms of the proliferation disease. The other symptoms, such as small fruit, may be caused by other disorders. Witches' brooms usually appear only during the first few years of the disease, but enlarged stipules can occur for up to 5 years following infection (Schmid, 1965). The witches' brooms or enlarged stipules may not occur for one or more years during the period of symptoms. Trees with masked infections will show symptoms again after severe pruning.

A partial recovery of infected trees as well as the total disappearance of symptoms can take place (Schmid, 1965). Fruit size may start to increase a few years following new infections, if these new infections do no occur within eight years after tree planting. However, if infection

of a tree occurs early in its life, the tree will always grow poorly, and its fruit will remain small.

1.2.4 - Transmission

Certainly the phytoplasma can be transmitted from tree to tree by grafting buds or scions. Colonization by the pathogen is irregular in the aerial parts of infected tree, and varies during the years. Thus, graft transmission tests may vary unless root pieces are used as inoculum (Kunze, 1989).

Pruning cut, slash or the contact with pruning shears used on infected plant, are not risky for the propagation of the disease. Again, the pathogen is not transmitted through pollen or seed (Seidl and Komarkova, 1974).

Seemingly the root bridges seem to allow the phytoplasmal transmission in healthy plant (Vindimian *et al.,* 2002).

Doubtless, the spread of the disease occurs mainly by means of insect vector. Psyllids seem to play a crucial role in the transmission of phytoplasmas to the Apple Proliferation phylogenetic cluster. *Cacopsylla pyricola* Förster and *Cacopsylla pyri* Linnaeus have been reported as vectors of *Ca.* Phytoplasma pyri, whereas *Cacopsylla pruni* Scopoli is able to transmit *Ca.* Phytoplasma prunorum (Jensen *et al*., 1964; Lemoine, 1991; Carraro *et al*., 1998; Carraro *et al*., 2001).

For *Ca.* Phytoplasma mali, psyllids seem to be chiefly responsible for the transmission of the disease, even though leafhoppers also have been reported as possible vectors.

Hegab and El-Zohairy (1986) described the ability of a spittlebug, *Philaenus spumarius* Linnaeus (Homoptera: Cercopidae) and a leafhopper, *Artianus interstitialis* Germar (Homoptera: Cicadellidae), in transmitting *Ca.* Phytoplasma mali from infected celery to apple seedlings and from infected to healthy celery. Another leafhopper, *Fieberiella florii* Stål (Homoptera: Cicadellidae), has been implicated in unconfirmed reports as a vector of *Ca.* Phytoplasma mali in Germany (Krczal *et al.*, 1988).

Frisinghelli *et al.* (2000) and Jarausch *et al.* (2003) reported that the psyllid *Cacopsylla picta* Förster (synonym *Cacopsylla costalis*) is a vector of *Ca.* Phytoplasma mali in Northeastern Italy and in Germany, respectively, while in Northwestern Italy *Cacopsylla melanoneura* Förster is the vector of the patoghen (Tedeschi *et al.*, 2002).

1.2.5 - Diffusion and situation of *Ca*. Phytoplasma mali in Trentino

Ca. Phytoplasma mali is spread in all Countries of Central and Southern Europe and its infectious nature is well-known in Trentino since 1950 (Rui, 1950).

The extent and intensity of the disease is variable in time therefore the absence or poor presence of symptoms for a few years does not guarantee the absence of phytoplasma.

In Trentino the first focus was registered in the mid-sixties in the area of Lake Garda. Following foci occurred in the eighties while after the nineties the disease spread with considerably greater frequency, in particular, in the area of Val di Non and Val di Sole where it struck down all cultivars grafted on various rootstocks (Vindimian & Delaiti, 1996). These valleys are in Northwestern Trentino and they are characterised by specialized horticulture of considerable tradition that supplies a high quality product.

Apple growing in these areas extends to about 1600 ha distributed between 400 and 1000 m a.s.l.

Three years of research conducted in orchards of these valleys shows a exponential growing of the disease. The percentage of infected plants was 0,8% in 1999, 1,7% in 2000 and 5,5% in 2001.

Furthermore, the oldest plants (more than 20 years) often show the AP symptoms, in particular if they are grafted on vigorous rootstocks. AP has become a serious epidemic and an economic problem in apple orchards, considering that 30% of the fruit orchards of Val di Non and Val di Sole are more than 20 years old.

Again, the percentage of infected plants is higher in orchards that grow over 800 m a.s.l. It is possible that the live woods near the apple growing areas include some host plants where the insect vectors overwinter.

1.3 - PSYLLIDS

Psyllids or “jumping plant lice” are small phytophagous, phloem feeding insects that are typically monophagous (feed on a single plant) or oligophagous (feed on a few related plants). Only a few are polyphagous, for example *Bactericera nigricornis* Förster. The adults are often able to exploit other “food-plants” temporarily.

Together with aphids, coccids and whiteflies they form the monophyletic group, Sternorrhyncha (Homoptera), which is considered basal within the true bugs (Hemiptera).

Psyllids are probably the most benign of these four insect groups and therefore the least well studied. “Sternorrhyncha” refers to the rearward position of the mouthparts relative to the head.

They are usually only 2-3mm long and bearing a strong resemblance to miniature cicadas. These insects are found particularly on trees and are not numerous. Many species keep to one particular kind of tree - a glance at a check list will reveal Alder Psyllid, Apple Psyllid, Birch Psyllid, and many others - and several are responsible for gall formation. The shape and resting attitude of the insects are quite characteristic, but the most diagnostic feature is the venation of the forewings. The forewings are somewhat thicker than the hind wings and may or may not be mottled. Psyllids do not fly well, but they are excellent jumpers and they are commonly known as jumping plant lice (Chinery, 1993).

1.3.1 - Evolution

Psyllid fossils have been found from the early Permian, before the angiosperms evolved. Thus, psyllids may have primitively fed on gymnosperms, or even lycopods (Hodkinson, 1980). The explosive radiation of the angiosperms in the Cretaceous was paralleled by a massive radiation of associated insects, and many of the morphological and metabolic characters that the angiosperms exhibit may have evolved as defenses against herbivorous insects. Primitively, insects were probably saprophagous with a shift to the more complex lifestyle of herbivory, a secondary adaptation (Mitter *et al.*, 1988). Allocation of resources in plants can vary seasonally, within an individual plant, between individuals, and from species to species. An example of this is the phenological changes in chemical profile of flowers, leaves, stems, and fruit for the legume species, Adenocarpus (Greinwald *et al*., 1992). The changing character of an individual plant is a complex and challenging landscape to herbivorous insects (Wink, 1992). An insect that attains an adaptive peak on one plant species is likely to be in an adaptive trough on another species (Janzen, 1979).

1.3.2 - Reproduction and ecology

Most psyllids develop on the young shoots, leaves, and leaf-stalks, others (e.g. pear psyllids) also on woody twigs and branches, and others (e.g. *CraspedoIepta sonchi* Förster) on the subterranean parts. Some psyllid species cause cecidia or other malformations in shoots *(Livia),* on leaves, e.g. *Psyllopsis fraxini* Linnaeus and *Psyllopsis discrepans* Flor,

Trichochermes walkeri Förster, etc, or on roots (*Neocraspedolepta subpunctata* Förster) (Ossiannilsson, 1992).

Almost all Psylloidea are normally bisexual. An exception is *Cacopsylla myrtilli* Wagner, an example of facultative parthenogenesis. Most psyllids (exception: *Livia)* place their eggs on the exposed surface of the leaves, shoots, stalks, etc, of the host-plant. The pedicel of the egg is inserted into the plant tissue. In some species a shallow "pit gall" appears as early as during oviposition. Then each young first instar nymph usually remains in its pit until its first moult. After that it usually moves to another part of the plant. The nymphs activity causes the leaves to swell and roll and the veins to turn red, and the psyllids continue to grow inside these galls. The flat, greenish nymphs, their stages recognised by the increasing number of antennal segments, are covered with fluffy wax threads and look like tiny pieces of cotton wool (Chinery, 1993).

The number of nymphal instars is five. The species can be univoltine, with one generation per annum; examples of bivoltine species, with two generations p.a., are *C. pyricola* and C. *pyri* , and *Trioza chenopodii* Reuter. In countries with warmer climatic conditions, many species are polyvoltine, producing several broods every year.

Most of psyllids hibernate in the adult stage, a few on their host-plants (*C. pyricola*) but most species on "shelter plants", usually conifers, or in crevices in bark or other protected sites. Whether psyllids hibernating on conifers do actually feed on them, is still unknown. In spring these species seek out their host-plants to mate and oviposit. Some psyllids (e.g. *Psylla* spp., *Cacopsylla mali* Schmidberger, *Cacopsylla sorbi* Linnaeus, *Cacopsylla peregrine* Förster, *Cacopsylla hippophaes* Förster) hibernate in the egg-stage on their host-plants, and a few species (*C. sonchi)* in a nymphal instar, also on the host-plant.

Stridulation has been observed in many psyllids (Heslop-Harrison, 1961; Campbell, 1964; White, 1970; Yang *et al.*, 1986). Rapid wing-vibrations are generally associated with the sound-production, but the exact mechanism has not been definitely established. Taylor (1985) described a possible stridulatory organ consisting of "rows of teeth on the axillary cords of the mesoscutellum and metascutellum, with corresponding rows of teeth under the second anal veins of both wings". Apparently this sound-production - or certain calls - plays a role in courtship.

The liquid excreta of psyllids contain sugar, and dense populations of psyllid larvae are often visited by ants, flies and bees to collect their honeydew. The circumanal pore-rings of adults and nymphs produce wax more or less copiously. Additional wax glands may al so be present on the integument of nymphs, especially on the abdomen. In the nymphs of Triozidae, each

sectaseta produces a long white wax filament. In other forms, flocculent masses of loose wax powder are produced, apparently protecting the insects from desiccation and also from the moisture of their honeydew.

1.3.3 - Psyllid damage

During their feeding, psyllid nymphs may cause considerable damage to their host-plants since their salivary injections may produce serious necroses, galling and malformations. Furthermore the high sugar concentration of honeydew produced by psyllid larvae can eat into the leaves of infested plant.

Certain species are vectors of phytoplasmal or viral diseases. Exactly when the stylets reach a phloem tube the insect injects saliva into the plant cell. The introduction of saliva allows the transfer of phytoplasmas or plant virus.

1.3.4 - Psyllids vector of *Ca.* Phytoplasma mali

There are two species of Psyllids involved in transmission of *Ca.* Phytoplasma mali: *Cacopsylla picta* Förster, synonym *C. costalis* (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2003) and *Cacopsylla melanoneura* Förster (Tedeschi *et al.*, 2002). Both species are present in Trentino. They are described as follows.

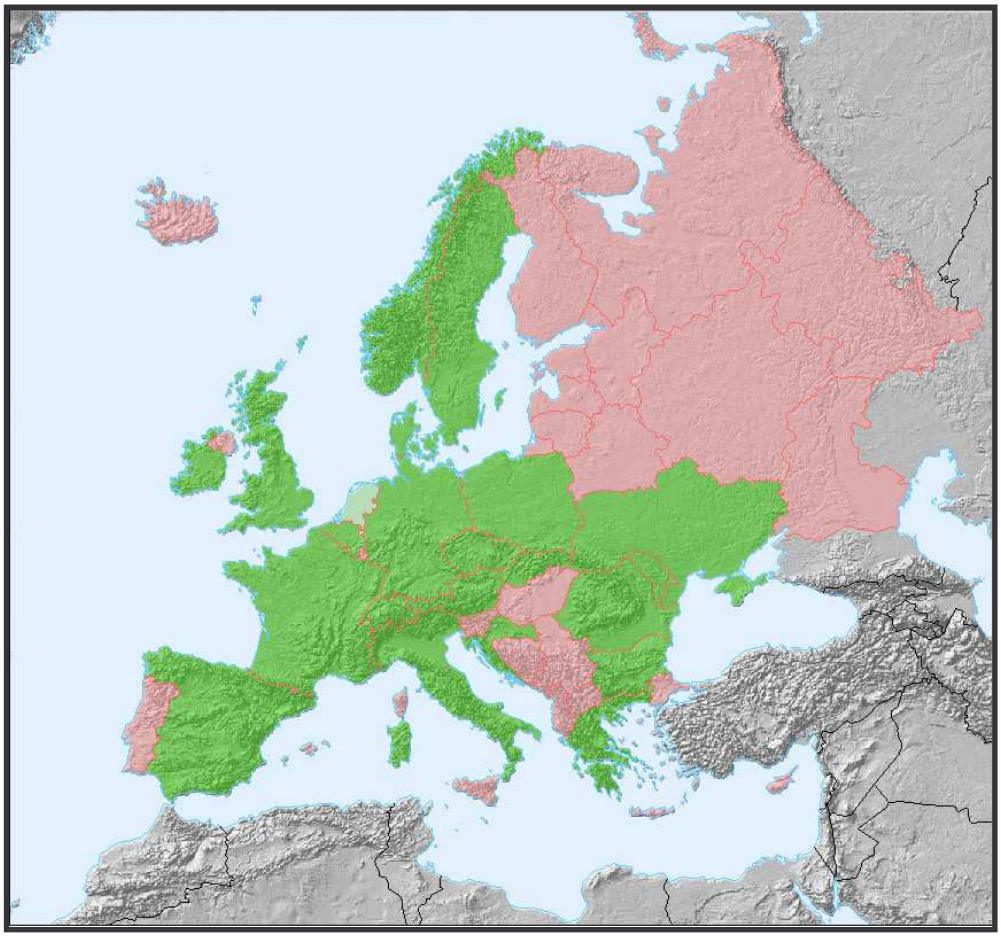
1.3.4.1 - *Cacopsylla melanoneura* Förster

A Holo-Palaearctic species is distributed everywhere with its host plants of the genus *Crataegus*. It is known in the whole of Europe (**Fig. 9**) except for the easternmost state. Occasionally, it has even shown up beyond the Polar Circle in Norway and Sweden (Ossiannilsson, 1992). The southern limit of its range runs through Spain, Italy, except for Sicily, Greece, Transcaucasia (Georgia, Armenia and Azerbaijan), Middle Asia (Tajikistan and Uzbekistan). Besides, it is known in Siberia, Dzungaria, Mongolia, China, the Russian Far East, and Japan. In the Czech Republic, especially in Moravia, this is one of the most abundant psyllid species. In Italy its presence is reported in Friuli Venezia Giulia, Lombardia, Piemonte and Val d'Aosta region and in the province of Trentino.

It produces one generation annually and hibernates in the adult stage (**Fig. 10**). *C melanoneura* is widely oligophagous on *Crataegus* spp. (C. *monogyna,* C. *oxyacantha,* C. *maximowiczii,* etc.). In some regions, like Trentino, there are populations which live on, and cause damage to apple trees *(Malus* spp.), occasionally also pear trees (*Pirus* spp.). In the

Crimea, members of the population which lives on apple trees will not-develop if transferred to hawthorn and die within several days (Lauterer, 1999).

Fig. 9: distribution of *C. melanoneura* in the European States (green = presence; green light = doubtful; pink = absence) (www.faunaeur.org/).

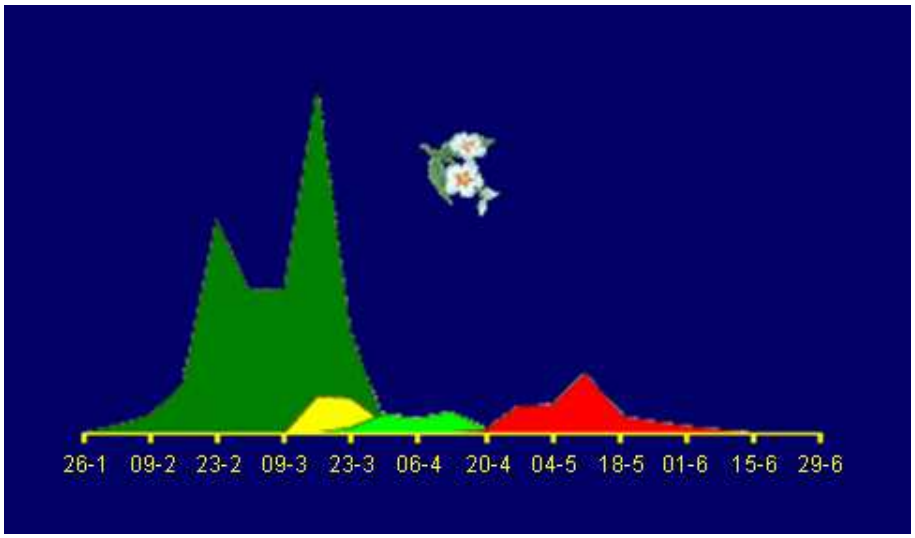


In Trentino the overwintering adults reappear on the apple trees from late January to early March. The females move in the orchards and start the oviposition with more or less intense activity. Embryonic development lasts 25-30 days and the first instar larvae start to gradually colonize the flower clusters. For another 20 days new larvae appear while simultaneously the first individuals change in nymphs. The insects complete 3 larvae and 2 nymphs instars before becoming adults that soon disappear from apple trees (**Fig. 11**). From observations conducted in the last years at the Istituto Agrario di San Michele all'Adige it seems likely that the insects hibernate in orchards.

Fig. 10: adult sample of *C. melanoneura*.

C. melanoneura frequently hibernates together with the group of salicicolous psyllids (the *C. saliceti* group) and with *C. affinis*. In its host plants it lives together with *C. affinis*, *C. peregrina*, and the phenologically delayed *C. crataegi* (Lauterer, 1999).

Fig. 11: development of *C. melanoneura* in Trentino. ■ Overwintering Adults, ■ eggs, ■ larvae and nymphs, ■ new generation adults.



1.3.4.2 - *Cacopsylla picta* Förster

This species is more commonly known under the name *C. costalis*. It has been synonymised with *C. picta* (**Fig. 12**). A European and Asia Minor species, very rare in the western and northern part of its range but, abundant in Bulgaria and Anatolia where it is a pest. It is not present in Great Britain. The species is known in isolated localities in southern Sweden and southern Finland, France, Switzerland, Germany, Austria, the Czech Republic, and Italy (**Fig. 13**); it is abundant in Bulgaria but extremely rare in most European countries. In Italy its presence is reported in the Friuli Venezia Giulia region and in the province of Trentino.

The species is monovoltine, hibernating in the adult stage. The species is narrowly oligophagous on *Malus domestica*, *M. sylvestris* and *Prunus armeniaca*. It hibernates chiefly on conifers (*Picea abies* and other species).

In Trentino the overwintering adults migrate to apple trees in spring between late March and mid-April. They are present for 40-50 days. The female lays the white-yellowish eggs separately both on young leaves at the top of the shoot not yet developed and on the under surface of young leaves in the niche provided. A female depose about 160 eggs. The oviposition is graduated and goes on for 30-40 days. The larvae appear for about one month and overlap to nymph, giving origin to a long period of young forms (two month). The new generation adults appear in early June until mid-July. After that, they disappear from apple trees (**Fig. 14**). It's also likely that *C. picta* hibernates in orchards.

The species exist together with *C. mali* in summer.

Fig.12: adult samples of *C. picta*.

Fig. 13: distribution of *C. picta* in the European States (green = presence; pink = absence) (www.faunaeur.org/).

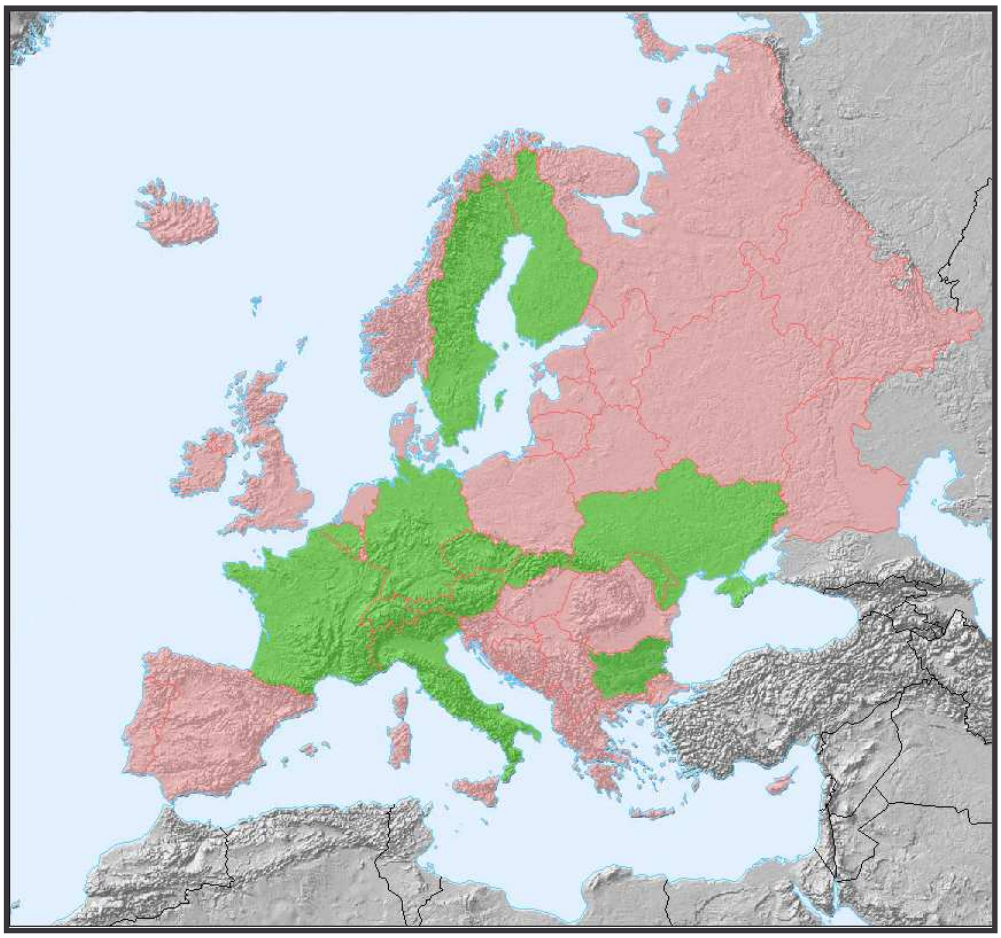
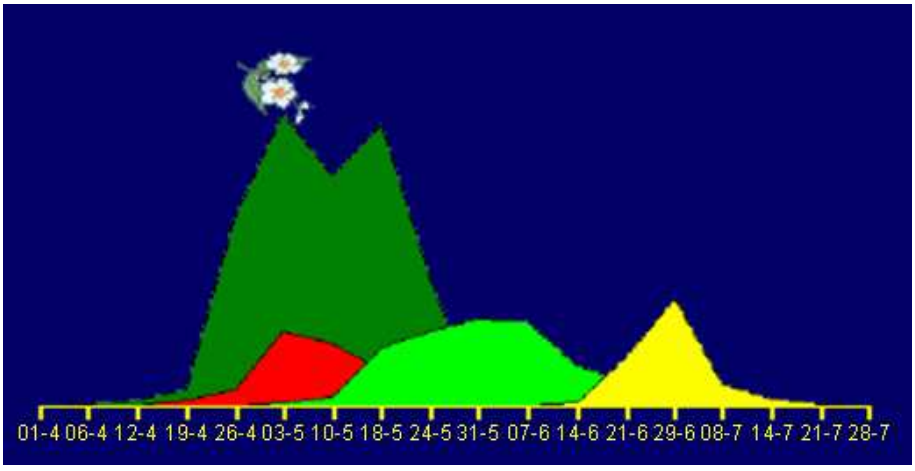


Fig. 14: development of *C. picta* in Trentino. ■ Overwintering Adults, ■ eggs, ■ larvae and nymphs, ■ new generation adults.



1.4 - APHIDS

Aphids, also known as greenfly, blackfly or plant lice, are minute plant-feeding insects in the superfamily Aphidoidea in the homopterous division of the Hemiptera order. Recent classification within the Hemiptera has changed the old term “Homoptera” to two suborders: Sternorryncha (aphids, whiteflies, scales, psyllids...) and Auchenorryncha (cicadas, leafhoppers, treehoppers, planthoppers...).

About 4,000 species of aphids are known, classified in 10 families; of these, around 250 species are serious pests for agriculture and forestry as well as an annoyance for gardeners. They vary in size from 1-10 mm long.

Aphids are distributed world-wide, but they are most common in temperate zones. It is possible for aphids to migrate great distances (mainly through passive dispersal riding on winds) depending on the weather patterns; for example, the lettuce aphid spreading from New Zealand to Tasmania. They have also been spread by human transportation of infested plant materials.

Twenty-five percent of all plant species are infested with aphids, and though it is believed that the speciation of aphids has followed that of plants, not all groups of plants are equally parasitised. Compositae, the 3rd most specious plant family, supports most aphid species (605 species), but the Orchidacea, the 2nd most specious plant family, only supports 9 species of aphids while the Rosaceae which is only the 22nd most specious plant family supports the 3rd highest number of aphid species (293 species). The plant family which supports the 2nd highest number of aphids is Coniferae (363 species) but these are non-flowering plants (Kennedy and Stroyan, 1959).

1.4.1 - Anatomy

The most typical organ of aphids is their piercing-sucking mouthparts called stylets. They have soft bodies; long, thin legs; two-jointed, two-clawed tarsi; and usually a pair of abdominal tubes through which a waxy secretion is exuded. These tubes were formerly supposed to secrete the sweet substance known as "honeydew" so much sought after by ants; but this is now known to come from the alimentary canal.The wings when present are two pairs, lacy, transparent and only have one prominent longitudinal vein. Aphids also have a proboscis originating between and behind the forelegs. Aphids' antennae are composed of two

thick basal segments and a flagellum with as many as four segments. The last of these four segments is divided into a proximal part and a thinner distal part (called process terminalis).
Aphids have two compound eyes and two ocular tubercles made up of three lenses, each of which is located behind and above the compound eyes. They have two tarsal segments. The fifth abdominal segment bears a pair of tubes on the dorsal surface named siphunculi (cornicles), which are upright and point backward. A cauda is usually present below and between them on the last abdominal segment (en.wikipedia.org/wiki/Aphid).

1.4.2 - Diet and feeding ecology

Most aphids are monophagous (i.e. feeding only on 1 species of plant). Others, like *Myzus persicae* feed on hundreds of plant species across many families.
Aphids passively feed on sap of phloem vessels in plants. This sap being kept under high pressure, once a phloem vessel is punctured, is forced into the food canal. Aphids actively "drink" (suck) from xylem vessels when thirsty. As they feed, aphids often transmit plant viruses to their food plants.
They tap into the plants with the stylets of their proboscis. They gain access to the phloem vessels from 3 main parts of the plant, stems, leaves, and roots. Their stylets, which are contained within the proboscis when the aphid is not feeding, are very thin and could suffer damage while being pushed into the plant or bend in an unwanted direction. Therefore aphids secrete a special liquid from the tips of their stylets which starts to harden as soon as it leaves the stylets forming a hard protective sheath around the stylets as they are slowly pushed into the plant in search of the phloem tubes. When the stylets reach a phloem tube the aphid injects saliva into the plant cell. It is suspected, but not known for sure, that this saliva helps prevent the plant cell from sealing the puncture (i.e. the aphids mouthparts) with special proteins. These special proteins are the plants normal defence mechanism. They are deposited on the wall of the cell around a puncture as a result of the drop in redox potential that occurs along the cell wall following puncture damage. Aphids insert their stylets slowly and it takes quite a bit of time to tap into a phloem tube, it can be anywhere from 25 minutes to 24 hours from starting to insert the stylets to actually getting something to eat.
Some aphids always feed by inserting their stylets through the stomata of the plant leaves they are feeding on. Other aphids penetrate to the phloem either intracellularly (pushing the stylets between cells), intercellularly (pushing the stylets through cells) or by a bit of both. Plant phloem saps are rich in sugars and poor in amino-acids or nitrogen. This results in aphids

excreting large amounts of sugary liquid, the honeydew, because the amount of sap they have to drink in order to get enough nitrogen in their diet means they have far more sugar and liquid than they need. This honeydew can often be seen on the lower leaves of infested trees where it falls giving them a sticky coating. This is then fed on by other insects like some species of ants, the common wasp *Vespula vulgaris* Linnaeus and the Brown Hairstreak Butterfly *Thecla betulae* Linnaeus and a yeast-like fungus which makes the leaves look all black as if they were covered with soot.

Nearly all aphids contain endosymbionts in special groups of cells called bacteriocytes. In most aphids the main endosymbiont is *Buchnera aphidicola*. It is believed to complement the aphids diet by synthesising vitamins, sterols and certain amino acids that are absent in the phloem. Whatever their role they are important for the aphids growth and reproductive potential as they decrease as the aphid gets older and are absent from most soldiers and males. The relationship between Buchnera and the aphids is obligatory. The bacteria can't live outside of the aphids and the aphids can't reproduce successfully without it. Buchnera is transmitted maternally via the ovary, new aphids get the symbiont from their mums when they are just an egg.

Most aphids are autoecious (living on one or a few species of closely related plants). About 10% are heteroecious spending autumn, winter and spring on one plant species (its primary host) and summer on a different unrelated plant (its secondary host). For example the Rosy Apple Aphid *Dysaphis plantaginea* has *Malus* sp. as its primary host and *Plantain Plantago lanceolata* as its secondary or summer host. Some heteroecious aphids such as *Myzus persicae* the Peach or Potatoe Aphid and *Aphis fabae* the Black Bean Aphid have a wide range of secondary hosts, but this is relatively rare. Most heteroecious aphids have just one primary and one secondary host (Auclair, 1963).

1.4.3 - Reproduction

Apart from their importance from the economic standpoint, aphids are chiefly remarkable for the phenomena connected with the propagation of the species. For part or all of their life, most aphids are often found to be parthenogenetic. Aphids have been known to have what is called telescoping generations. With telescoping generations the female aphid will have a daughter within her who is already parthenogenetically producing its own daughter at the same time. This leads to the bizarre situation where the diet of a female aphid can have inter-generational effects on the body size and birth rate of aphids. In other words, what the aphid

eats can directly change the size and fertility of the aphid's daughters and grand-daughters (Nevo and Coll, 2001; Jahn *et al.*, 2005).

At different times of the year, they can be viviparous or oviparous. During spring and summer, aphids are often parthenogenetic and viviparous and then give birth sexually during autumn. Therefore aphids are said to undergo “cyclical parthenogenesis” or to have a “holocyclical” life circle.

Male and female aphids mate in autumn. Sexual females, but also asexual ones, have two sex chromosomes while sexual males only have one. However, in reality sperm with a kind of sex chromosome degenerate very rapidly and never contribute to an embryo.

Eggs produced in the autumn by fertilized females remain on the plant through the winter and hatching in the spring give rise to female individuals which may be winged or wingless. From these, females are born parthenogenetically: that is to say, without the intervention of males, and by a process that has been compared to internal budding. Large numbers of young resembling their parents in every respect, except in size, are produced. They themselves reproduce their kind in the same way. This process continues throughout the summer, generation after generation being produced until the number of descendants from a single individual of the spring-hatched brood may amount to many thousands. In the autumn winged males appear; union between the sexes takes place and the females lay the fertilized eggs which are destined to carry the species through the cold months of winter. If, however, the food-plant is grown in a glasshouse or greenhouse where protection against cold is afforded, the aphids may go on reproducing agamogenetically (asexually) without cessation for many years. Likewise, in warm and tropical areas or during the growing season, aphids reproduce asexually without interruption. Since the young can become adults and reproduce within a few days, this process leads to the building-up of very large populations responsible for severe damage to crops and important economic losses; such populations often require pest control. Interesting features connected with this strange life-history are the facts that the young may be born by the oviparous or viviparous methods and either gamogenetically or agamogenetically. These may develop into winged forms or remain wingless, and the males only appear at the end of the season. Although the factors which determine these phenomena are not clearly understood, it is believed that the appearance of the males is connected with the increasing cold of autumn and the growing scarcity of food, and that the birth of winged females is similarly associated with decrease in the quantity or vitiation of the quality of the nourishment imbibed. Sometimes the winged females migrate from the plant they were born on to start fresh colonies on others often of quite a different kind (host plant alternancy). Thus

the apple aphid (*Aphis mali*) after producing many generations of apterous females on its typical food-plant gives rise to winged forms which fly away and settle upon grass or corn-stalks (Dixon, 1977).

1.4.4 - Evolution

Aphids probably first appeared 280 million years ago, in the Carboniferous period. They probably fed on non-flowering plants like Cordaitales or Cycadophyta. The oldest known aphid fossil is one of the species Triassoaphis cubitus from the Triassic. There were relatively few species of aphids at that time, and the number of species only considerably increased since the appearance of angiosperms 160 million of years ago. This is due to the fact that angiosperms provide an occasion for aphids to become specialized.

Aphids have not always looked like they do nowadays. Organs like the cauda or the siphunculi were not evolved until period of the Cretaceous (en.wikipedia.org/wiki/Aphid).

1.4.5 - Aphids as Pests

Aphids are pests and same of them are considered harmful to plants. They are pests firstly, because their feeding reduces the vitality of the crops they feed on and secondly, because they often transmit viral diseases. It has never been demonstrated that an aphid is a vector of phytoplasmas.

Currently, the aphid pests control still involves large amounts of pesticides in some countries, but other more ecologically friendly methods have been used in other places for some time. These generally involve biological control, mostly with a range of Hymenopteran parasites, or genetic manipulation for aphid resistance (www.earthlife.net/insects/aphids.html).

1.4.6 - Main aphids analysed

Five species of apple aphids were analysed for phytoplasma detection: green apple aphid (*Aphis pomi* De Geer), rosy apple aphid (*Dysaphis plantaginea* Passerini), woolly aphid (*Eriosoma lanigerum* Hausmann), rosy leaf-curling aphid (*Dysaphis devecta* Walker) and apple-grass aphid (*Rhopalosiphum insertum* Walker). The three main species are described as follows.

1.4.6.1 - Green apple aphid (*Aphis pomi* de Geer)

1.4.6.1.1 - Description

- Apterous adult: 1,5-2,0 mm long; body oval and relatively rounded; green with black siphunculi and cauda; legs and antennae paler and tipped with brown (**Fig. 15**).
- Winged adult: thorax black; abdomen green with 3 pairs of black lateral circular spots on the anterior abdominal segments and a semicircular spot in front and behind each siphunculus.
- Egg: greenish-yellow to green, rapidly turning to shiny black.

1.4.6.1.2 - Biology

- Host plants: apple, more rarely pear, hawthorn (*Crataegus oxyacantha*), medlar (*Mespilus*), quince, rowan or mountain ash (*Sorbus*), rose (*Rosa*) and spiraea (*Spiraea*).
- Unlike most harmful aphids, *A .pomi* is an autoecious species.
- Number of female nymphs produced by oviparous females: 60.
- Nymph: fundatrix developing in 3 weeks.
- Aphids feed by inserting their stylets into the phloem. They often form compact sheaths on shoots, thousands of individuals sometimes being present. They infest the reverse of terminal leaves which roll and undergo moderate leaf curl.

1.4.6.1.3 - Life Cycle

- Winter eggs hatch after bud-burst and give rise to fundatrices which are apterous parthenogenetic viviparous females producing a generation of viviparous parthenogenetic females.
- Ten to 15 generations succeed one another from spring to autumn.
- Winged forms appear from April onwards, migrating to other trees on which they deposit their eggs; light winds enable them to move more easily, carrying them to a distance of up to several dozens of kilometers.
- Winged oviparous females and apterous males appear in October and November. After mating, each female lays eggs on the twigs, preferably at the top of current year's growths. These eggs are sometimes grouped in large numbers, unlike those of other aphid pests.

1.4.6.1.4 - Damage

- Feeding punctures of fundatrices and their offspring hinder growth of the young twigs of host trees and sometimes distort them. Damage is more severe on nursery stock and seedlings.
- In summer, sooty moulds develop on the honeydew which sometimes is produced in large quantities.

(www.inra.fr/internet/Produits/HYPPZ/RAVAGEUR/6aphpom.htm)

Fig. 15: samples of *A. pomi*.

1.4.6.2 - Rosy apple aphid (*Dysaphis plantaginea* Passerini)

1.4.6.2.1 - Description

- Apterous adult: large aphid of 2,5 mm long; globe-shaped; purplish-olive-green to mauve; covered with a whitish pubescence (**Fig. 16**).
- Winged adult: dark green with a shiny mottle at the middle of the abdomen; siphunculi long and dark brown; cauda very short and distinctly conical.
- Egg: elongate and black.

1.4.6.1.2 - Biology

- This aphid is harmful only to apple; its secondary host plant is plantain, especially *Plantago lanceolata*.

- Winter eggs are deposited in autumn at the base of buds or under the bark. Each of the eggs hatch when buds swell to produce a fundatrix, which gives birth parthenogenetically to about 70 apterous virginoparae. Dense colonies develop on the underside of leaves or on twigs. Apterous virginoparae produce apterous sexuparae, winged sexuparae and males. Alatae grow progressively in numbers and migrate to plantain. Spreading of apterous forms on other trees is ensured by the wind.

1.4.6.2.3 - Life Cycle

- There are 6-9 generations per year.
- On apple, colonies develop in April, during the blossom period.
- Alatae appear in late May, migrating to plantain up to late July. Winged adults return to apple trees from late September to November and produce winter eggs, which represent the overwintering form.

1.4.6.2.4 - Damage

- This aphid is a very harmful species, causing severe distortion of plants. Leaves curl up and may drop prematurely, twigs become distorted and natural 'fruit drop' is impeded. The remaining fruits are small and bumpy. Vast quantities of honeydew are also produced and sooty moulds develop upon it.

(www.inra.fr/internet/Produits/HYPPZ/RAVAGEUR/6dyspla.htm)

Fig. 16: colony of *D. plantaginea*.

1.4.6.3 - Woolly aphid (*Eriosoma lanigerum* Hausmann)

1.4.6.3.1 - Description

- Apterous individuals: about 2 mm long; purplish-black, but this coloration is concealed by a woolly and filamentous wax covering, hence the name "woolly aphid" (**Fig. 17**).
- Alatae: body brown and lightly coated with a woolly covering; siphunculi pore-like and hardly visible.

1.4.6.3.2 - Biology

- Host plants: in America, the country from which it originates, this aphid has a sexual phase on its primary host, the American elm (*Ulmus americana*). In Europe, it reproduces exclusively parthenogenetically on apple. It is also to be found on quince but is rarely present on pear.
- Apterous nymphs and females lie overwinter under bark, in crevices in the trunk of trees, cankers or on roots near the collar. Activity resumes in early spring (March-April) and females begin to reproduce, each producing more than 100 nymphs.
- Heavy infestations form dense whitish colonies. Adults and nymphs feed by sucking up sap from ligneous parts or tender shoots but never from leaves.
- Alatae appear from July onwards and migrate, thereby forming new colonies on other trees.

1.4.6.3.3 - Life Cycle

- The capacity of this aphid to multiply is very important: 10-12 generations succeed one another until the autumn.

1.4.6.3.4 - Damage

- Probing and the injection of toxic saliva cause blisters and cankers to appear which can reach the size of a walnut and interfere with the circulation of the sap. Affected trees decline and can be attacked by secondary pests.
- Biological control attempts were carried out at the beginning of this century, using the parasite specific to the woolly aphid: *Aphelinus mali* (Hymenoptera).

(www.inra.fr/internet/Produits/HYPPZ/RAVAGEUR/6erilan.htm)

Fig. 17: sample of *E. lanigerum*.

1.5 - QUANTITATIVE PCR

In the last few years the quantitative PCR (q-PCR or real-time PCR) has been used for phytoplasmal diagnostic analysis. It offers great advantages as regards the analysis sensibility and specificity, reducing the risk of contamination. Furthermore, once a valid method is ready, the q-PCR offers a quantitative approach to research of connections between the phytoplasma, the host plant and the insect vector. Hence, it was proposed for Apple Proliferation disease (Baric and Dalla Via, 2004), Chrysanthemum Yellows disease (Marzachì and Bosco, 2005) and for phytoplasmas of different taxonomic groups (Christensen *et al.*, 2004).

1.5.1 - What is q-PCR?

In conventional PCR, the amplified product, or amplicon, is detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. In contrast, q-PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in "real time".

Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional

increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle.

The main advantage of q-PCR over conventional PCR is that q-PCR allows to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA).

Additionally, q-PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a closed-tube system, opportunities for contamination are reduced and the need for postamplification manipulation is eliminated.

1.5.2 - How q-PCR works

To understand how q-PCR works, let's start by examining a sample amplification plot (**Fig. 18**). In this plot, the PCR cycle number is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, is shown on the y-axis.

The amplification plot shows two phases, an exponential phase followed by a nonexponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28-40 in **Fig. 18**).

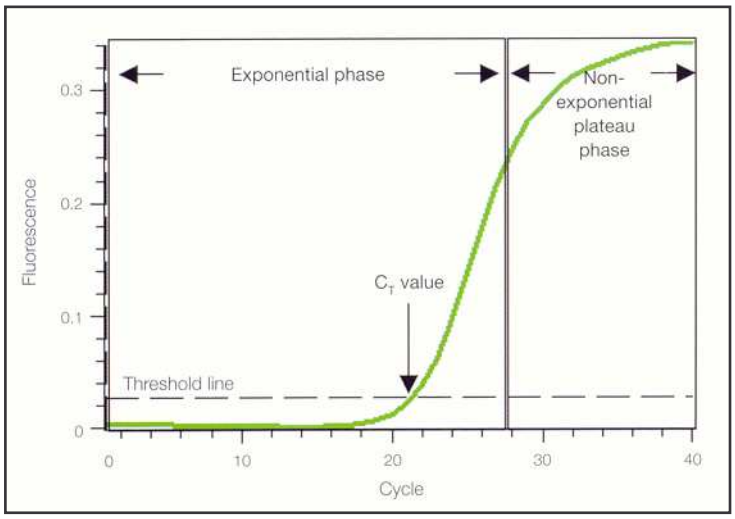
Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1-18 in **Fig. 18**) even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or Ct. Since the Ct value is measured in the exponential phase when reagents are not limited, q-PCR can be used to reliably and accurately calculate the initial amount of template present in the reaction.

The Ct of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction,

relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background.

Thus, the reaction will have a low, or early, Ct, In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, Ct. This relationship forms the basis for the quantitative aspect of q-PCR.

Fig. 18: amplification plot. Baseline-subtracted fluorescence is shown (Real-Time PCR Applications Guide - Bio Rad, 2005).



1.5.3 - Hallmarks of an optimized q-PCR assay

Since real-time quantification is based on the relationship between initial template amount and the Ct value obtained during amplification, an optimal q-PCR assay is absolutely essential for accurate and reproducible quantification of the sample. The hallmarks of an optimized q-PCR assay are:

- Linear standard curve ($R^2 > 0.980$ or $r > |-0.990|$).
- High amplification efficiency (90-105%).
- Consistency across replicate reactions.

A powerful way to determine whether q-PCR assay is optimized is to run serial dilutions of a template and use the results to generate a standard curve. The template used for this purpose can be a target with known concentration (e.g., nanograms of genomic DNA or copies of plasmid DNA) or a sample of unknown quantity (e.g., cDNA). The standard curve is

constructed by plotting the log of the starting quantity of template (or the dilution factor, for unknown quantities) against the Ct value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2), can then be used to evaluate whether your q-PCR assay is optimized.

Ideally, the dilution series will produce amplification curves that are evenly spaced, as shown in **Figure 19 A**. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the Ct values of the curves).

For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, n = 3,32, and the Ct values should be separated by 3,32 cycles. Evenly spaced amplification curves will produce a linear standard curve, as shown in **Figure 19 B**. The equation and r value of the linear regression line are shown above the plot.

The r or R^2 value of a standard curve represents how well the experimental data fit the regression line, that is, how linear the data are. Linearity, in turn, gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template copy numbers. A significant difference in observed Ct values between replicates will lower the r or R^2 value.

A good analysis must shows an r whose absolute value is >0,990 or an R^2 value >0,980 for the q-PCR reactions.

Amplification efficiency, E, is calculated from the slope of the standard curve using the following formula:

$E = 10^{-1/slope}$

Ideally, the amount of PCR product will perfectly double during each cycle of exponential amplification; that is, there will be a 2-fold increase in the number of copies with each cycle. This translates to a reaction efficiency of 2. Using an efficiency equal to 2 in the equation above, $2 = 10^{-1/slope}$, indicates that the optimal slope of the standard curve will be -3,32. Note that the absolute value of the slope is the same as the ideal spacing of the fluorescent traces described above.

Amplification efficiency is also frequently presented as a percentage, that is, the percent of template that was amplified in each cycle. To convert E into a percentage:

% Efficiency = (E-1) x 100%

For an ideal reaction:

% Efficiency = (2-1) x 100% = 100%.

For the example shown in **Figure 19**:

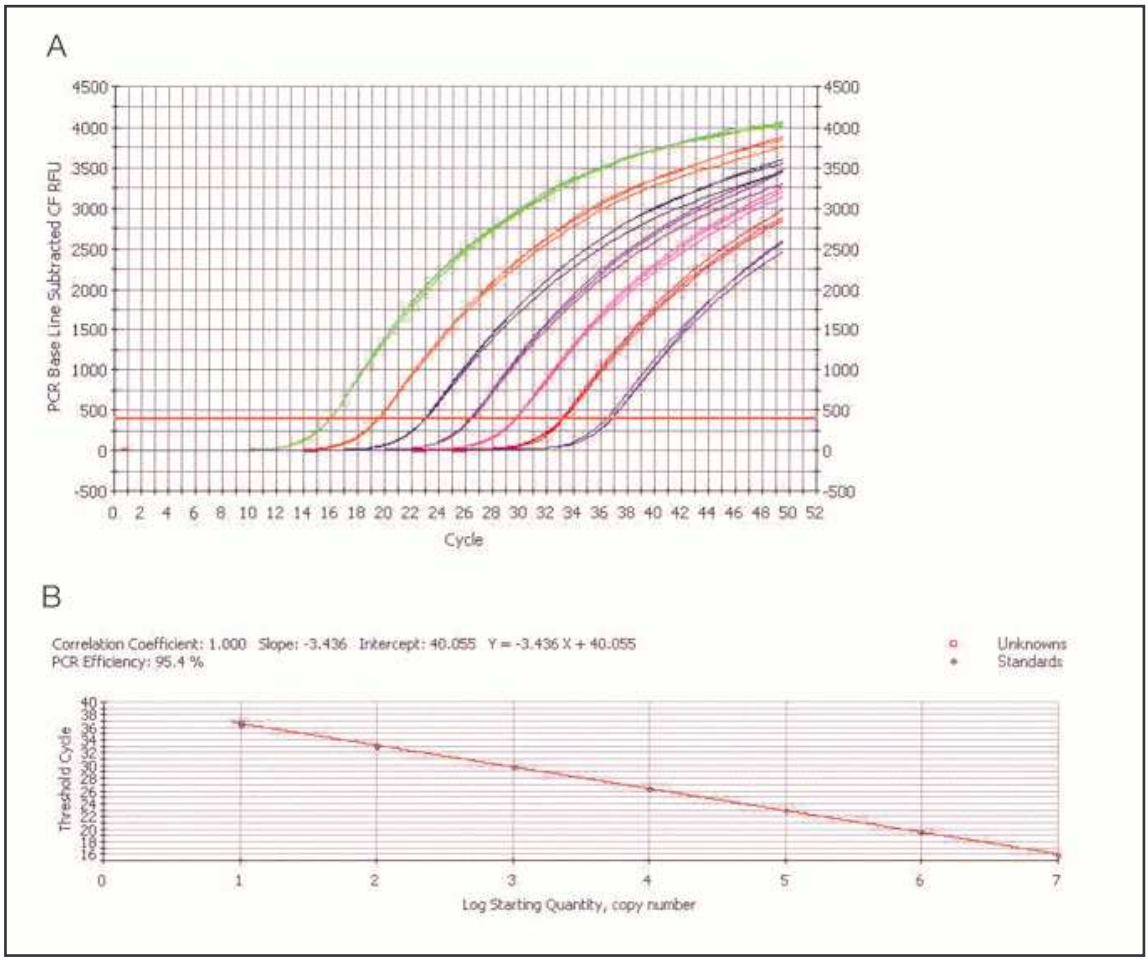
$E = 10^{-(1/-3,436)} = 1,954$

% Efficiency = (1,954-1) x 100% = 95,4%.

Fig. 19: generating a standard curve to assess reaction optimization. A standard curve was generated using a 10-fold dilution of a template amplified on a real-time system. Each dilution was assayed in triplicate.

A. Amplification curves of the dilution series.

B. Standard curve with the Ct plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph. The calculated amplification efficiency is 95,4% (Real-Time PCR Applications Guide - Bio Rad, 2005).



At the end of each cycle, the amplicon copy number increased 1,954-fold, or 95,4% of the template was amplified.

An efficiency close to 100% is the best indicator of a robust, reproducible assay. A good analysis must shows an amplification efficiency of 90-105%. Low reaction efficiencies may be caused by poor primer design or by suboptimal reaction conditions. Reaction efficiencies > 100% may indicate pipetting error in serial dilutions or coamplification of nonspecific products, such as primer-dimers. When using the method described above to determine amplification efficiency, the presence of inhibitor can also result in an apparent increase in efficiency. This is because samples with the highest concentration of template also have the highest level of inhibitors, which cause a delayed Ct, whereas samples with lower template concentrations have lower levels of inhibitors, so the Ct is minimally delayed. As a result, the absolute value of the slope decreases and the calculated efficiency appears to increase. If the reaction efficiency is <90% or > 105%, the assay must be modify by redesigning primers and probes.

1.5.4 - Chemistry selection

A key step in designing a q-PCR assay is selecting the chemistry to monitor the amplification of the target sequence. The variety of fluorescent chemistries available can be categorized into two major types:

- DNA-binding dyes (SYBR Green I);
- dye-labeled, sequence-specific oligonucleotide primers or probes (molecular beacons and TaqMan in particular).

The most commonly used chemistries for q-PCR are the DNA-binding dye SYBR Green I and TaqMan hydrolysis probes.

1.5.4.1 - DNA-Binding Dyes (SYBR Green I)

The most commonly used DNA-binding dye for q-PCR is SYBR Green I, which binds nonspecifically to double-stranded DNA (dsDNA). SYBR Green I exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1000-fold when it binds dsDNA (**Fig. 20**). Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified.

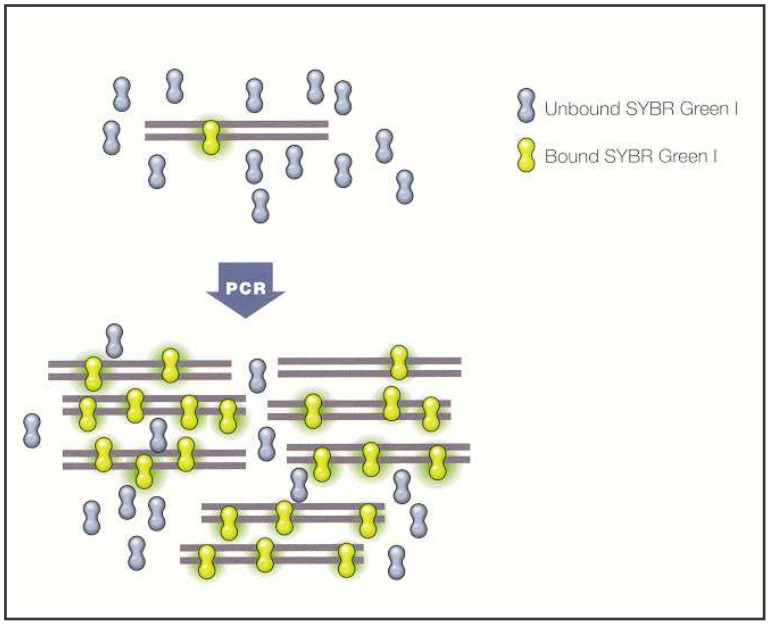
The advantages of using dsDNA-binding dyes include simple assay design (only two primers are needed; probe design is not necessary), ability to test multiple genes quickly without

designing multiple probes (e.g., for validation of gene expression data from many genes in a microarray experiment), lower initial cost (probes cost more), and the ability to perform a melt-curve analysis to check the specificity of the amplification reaction.

Melt-curve analysis can be used to identify different reaction products, including nonspecific products. After completion of the amplification reaction, a melt curve is generated by increasing the temperature in small increments and monitoring the fluorescent signal at each step. As the dsDNA in the reaction denatures (i.e., as the DNA "melts"), the fluorescence decreases. The negative first derivative of the change in fluorescence is plotted as a function of temperature. A characteristic peak at the amplicon's melting temperature (Tm, the temperature at which 50% of the base pairs of a DNA duplex are separated) distinguishes it from other products such as primer-dimers, which melt at different temperatures.

The major drawback of DNA-binding dyes is their lack of specificity, that is, DNA-binding dyes bind to any dsDNA. As a result, the presence of nonspecific products in a q-PCR reaction may contribute to the overall fluorescence and affect the accuracy of quantification. Another consequence is that DNA-binding dyes cannot be used far multiplex reactions because fluorescent signals from different amplicons cannot be distinguished. Instead, parallel reactions can be set up to examine multiple genes, such as a gene of interest and reference gene, in a q-PCR assay with SYBR Green I.

Fig. 20: DNA-binding dyes in q-PCR. Fluorescence dramatically increases when the dye molecules bind to dsDNA (Real-Time PCR Applications Guide - Bio Rad, 2005).



1.5.4.2 - Fluorescent primer- and probe-based chemistries

Many fluorescent primer- and probe-based chemistries have been devised and are available from different commercial vendors. The most commonly used probe- based chemistries are TaqMan probes.

Primer- and probe-based detection chemistries share some common features. In general, these chemistries take advantage of fluorescence resonance energy transfer (FRET), or some other form of fluorescence quenching, to ensure that specific fluorescence is detected only in the presence of amplified product.

The primer or target-specific oligonucleotide probe is labeled with a reporter fluorophore; but in most cases, the oligonucleotide is designed so that fluorescence is quenched when the specific target is unavailable. Usually this is accomplished by attaching a quencher molecule to the probe, and devising some mechanism by which the reporter and quencher are separated when the probe binds to its specific target.

In q-PCR, fluorescent primers and probes offer two main advantages over DNA-binding dyes. First, they specifically detect the target sequence so nonspecific products do not affect the accuracy of quantification. Second, they allow multiplex reactions to be performed.

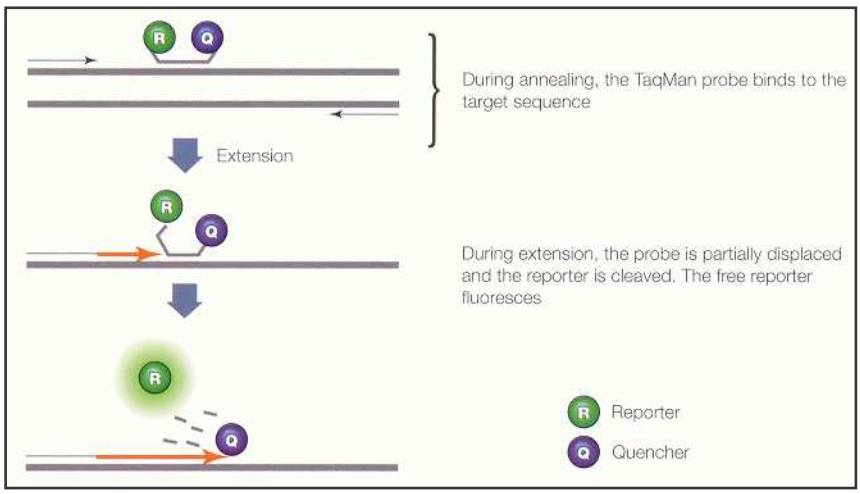
1.5.4.2.1 - TaqMan Assays

TaqMan assays employ a sequence-specific, fluorescently labeled oligonucleotide probe called the TaqMan probe, in addition to the sequence-specific primers.

Figure 21 illustrates how TaqMan assays work. Also known as the 5'-nuclease assay, the TaqMan assay exploits the 5'-exonuclease activity of certain thermostable polymerases, such as *Taq*. The probe contains a fluorescent reporter at the 5' end and a quencher at the 3' end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step of the amplification reaction, the probe hybridizes to the target and the dsDNA-specific 5'→3' exonuclease activity of *Taq* cleaves off the reporter. As a result, the reporter is separated from the quencher, and the resulting fluorescence signal is proportional to the amount of amplified product in the sample. One commonly used fluorescent reporter- quencher pair is fluorescein (FAM, which emits green fluorescence) and Black Hole Quencher 1.

The main advantages of using TaqMan probes include high specificity, a high signal-to-noise ratio, and the ability to perform multiplex reactions. The disadvantages are that the initial cost of the probe may be high and the assay design may not be trivial.

Fig. 21: TaqMan assay (Real-Time PCR Applications Guide - Bio Rad, 2005).



1.6 - MODEL REGIONS

In the project SMAP two model regions representing for Val di Non and Val d'Adige were selected respectively.

In Val d'Adige the orchards at **Piovi** belonging to the Istituto Agrario di San Michele all'Adige have been chosen as model region because these orchards have already been analysed and the history of the plantation is known. Furthermore, the plantation in 20 accurate blocs is ideal for subsequent statistical analyses. In 2005, 4 blocs randomly distributed in the area have been planted with new material. Although the planting material could not be tested for *Ca.* Phytoplasma mali infection prior plantation these blocs are otherwise ideal to follow the disease spread in the following years.

The core region of the model region of Piovi has a size of ca. 4 ha but 6 further blocs are considered (**Fig. 22-23-24**). Thus, the total surface is 8,2 ha. In 2005, the monitoring of the region has been extended to adjacent old orchards and therefore a total of 11,34 ha has been monitored for AP. In the core region psyllid captures have been done weakly from January to September in order to monitor the presence of psyllids in the orchards.

In September and October/November 2005 the region has been monitored twice for AP-infection by visual inspection. Maps of all orchards of the model region have been established. Each monitored tree received an identification code. With this code all data have been introduced into the SMAP database for analysis.

Fig 22: model region of Piovi (marked of red line) and Istituto Agrario di San Michele all'Adige (ringed in green) (maps.google.it/maps).

Fig. 23: enlargement of Piovi model region (maps.google.it/maps).

Fig. 24: model Region of Piovi.

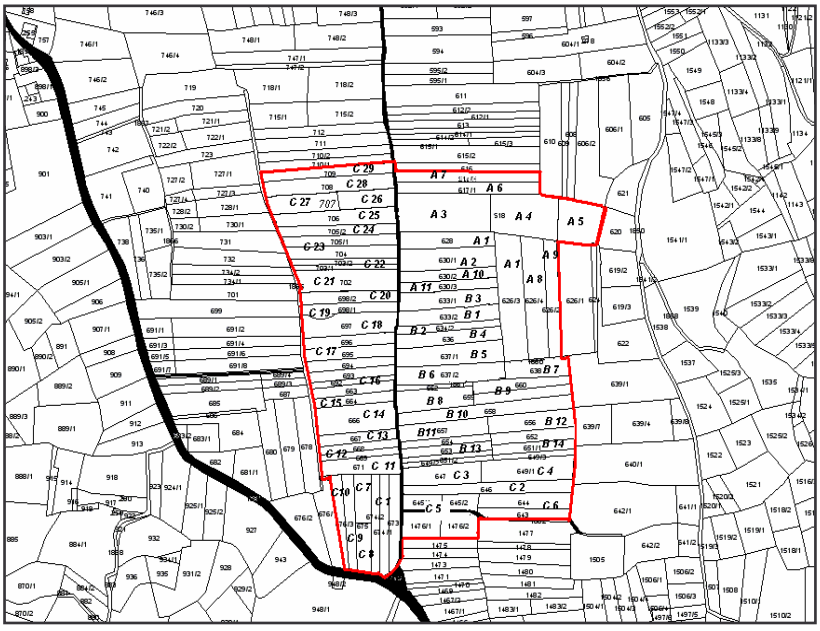
In Val di Non the model region has been chosen in the area of **Nanno**. This area is a historical apple growing region of Val di Non situated in the middle of the valley (in the Tuenno - Cles area). The selected model region is exclusively composed of private orchards but the composition of the area according to age of the orchards, cultivars and agricultural practices is typical of Val di Non. The model region is geographically homogenous and the distribution of the orchards should permit statistical analysis (**Fig. 25 A-B**).

Fig. 25: model region of Nanno.

A

B

C



The entire model region has a surface of 10,86 ha composed of 69 parts (**Fig. 25 C**) belonging to 37 owners. Uniform parcels can be defined which are planted with about 20.000 trees. The main cultivars are *Renetta del Canada*, *Golden delicious* and *Gala*. Within this area three core regions have been selected each composed of old, medium-aged and newly planted orchards. Like in Piovi the planting material of 2005 could not be tested for phytoplasma-infection prior plantation. Nevertheless, the new plantations of 2005 are sufficiently large to allow an analysis of the disease spread in the following years.

In September and October/November the core region has been monitored twice for AP-infection by visual inspection. In October/November the entire model region has been monitored.

Maps of the orchards in the model region have been established and each monitored tree received an identification code. With this code all data have been introduced into the SMAP database for analysis. All important data about the orchards have been collected.

1.7 - AIMS OF THE WORK

The main aim is the description and analysis of population dynamics of *Ca.* Phytoplasma mali present in apple trees and in insect vectors in orchard of some areas of Trentino

A definite situation of strain distribution can contribute to understand:

- the disease epidemic (How was the disease developed?);
- the possible specificity between phytoplasmal strain and the apple cultivar;
- the relation between strain, virulence and disease spread (Is the seriousness of the disease of these last years due to more virulent strain? Is there a strain with different virulence? What is its impact on the disease spread?).

That's why the following objectives were established:

- the molecular detection of *Ca.* Phytoplasma mali in plant and insect samples and the genetic variability analysis of pathogen;
- the distribution of three strains of phytoplasma in the territory;
- the research of possible correlations between the present strain and a set of parameters bound up with the plants, the insects and the territory such as the kind of cultivar and rootstock, the age of the plants, the developmental stages of vector and the area of sample

origin;
- The determination of the phytoplasmal titer in the sample relative to same parameters tested for the strain analysis;

A second aim of this research was to investigate the existence of further vectors by checking the presence of the pathogen in insect species and subsequently evaluating their capability of transmission. Some species of aphids which sucked on AP-infected trees were found in orchards and the work was focused on them. A high percentage of samples of aphids have been found *Ca.* Phytoplasma mali-positive by PCR.
To verify whether pathogen-infected aphids are able to transmit the phytoplasma to plants become another object of this research. Exactly, the quantification of phytoplasma in the aphids by q-PCR was carried out while the experimental transmission trials were conduct by Luisa Mattedi and Flavia Forno of the Plant Protection Department of the Istituto Agrario di San Michele all'Adige.

This work is a part of the project **SMAP** (Scopazzi del Melo - Apple Proliferation). This was funded by "Fondo Unico per i Progetti di Ricerca PAT" and tackled the problem of Apple Proliferation disease with interdisciplinary approach working in three ways:
- the development of a methodology more suitable to diagnose the disease, on the development of monitoring methods for the epidemiological studies and on the description of genetic variability of the pathogen in Trentino;
- the development of efficient vector control strategies;
- the development of resistant vegetable material starting from the apomictic rootstocks of agronomic value.

Partner of the project were the Istituto Agrario di San Michele all'Adige (IASMA - San Michele all'Adige - Italy), the RLP AgroScience GmbH (Neustadt - Germany), the AlPlanta Institute for Plant Research (IPR - Neustadt - Germany) and the Biologische Bundesanstalt (BBA - Dossenheim - Germany).

2 - MATERIALS AND METHODS

2.1 - PLANT SAMPLE COLLECTION

During the period of three years leave samples were collected from 843 apple tree (*Malus x domestica Borkh.*), showing the symptoms of the disease, in 10 different areas of Trentino (**Tab. 3**; **Fig. 26**). Five significant sites are situated in the valleys of North-West Trentino, in Val di Sole and Val di Non, where intensive apple orchards are grown and have a high rate of disease plants. This entire territory can be considered a high infection zone. In the other five areas of Central and South Trentino apple growing is less widespread and the pathogen is less present. These areas are located in a low infection zone.

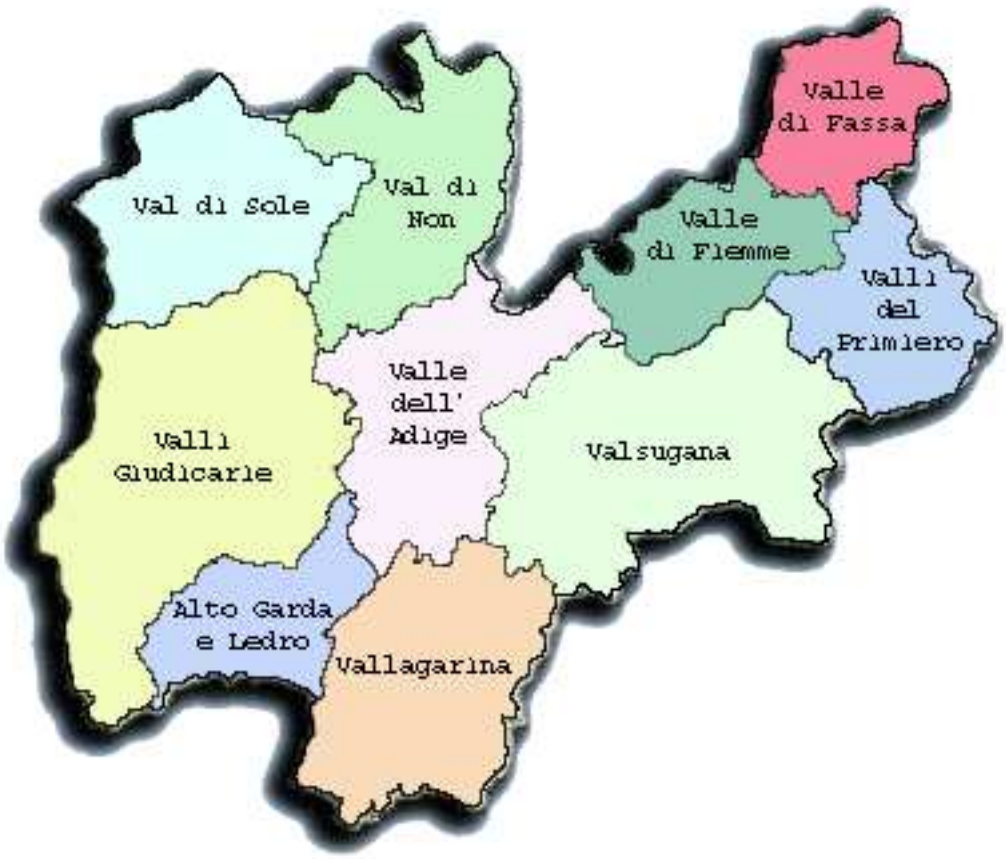
Tab. 3: the collection areas locate in several valleys of Trentino.

AREA	VALLEY
Val di Sole	Val di Sole
Revò - Alta Val di Non	Val di Non
Tuenno - Cles	Val di Non
Taio - Segno	Val di Non
Denno - Cuneo - Campodenno	Val di Non
Val d'Adige Nord	Val d'Adige
Val d'Adige Sud	Val d'Adige - Vallagarina
Valsugana	Valsugana
Val del Sarca	Alto Garda e Ledro
Bleggio area	Valli Giudicarie

Renetta del Canada and *Golden delicious* are the cultivars more consider. The first is present particularly in Val di Sole and Val di Non while the second is spread all over Trentino.
Every year plant samples were picked up in orchards and uncultivated fields from late September to early November when the disease symptoms were more manifested.
Samples were chosen considering the following variables:

- area of origin of the sample,
- cultivar of plant,
- type of rootstock,
- age of plant.

Fig. 26: Trentino map with the major valleys.



2.1.1 - First collection

In the first year 179 plant samples were representative of all areas (**Tab. 4**).

Tab. 4: plant samples collected in 2002.

AREA	CULTIVAR	N° SAMPLES
Val di Sole	*Renetta canada*	14
	Golden delicious	14
	Florina	2
Revò - Alta Val di Non	*Renetta canada*	12
	Golden delicious	14
	Gala	1
Tuenno - Cles	*Renetta canada*	15
	Golden delicious	10
	Florina	1
Taio - Segno	*Renetta canada*	10
	Golden delicious	10
Denno - Cunevo - Campodenno.	*Renetta canada*	8
	Golden delicious	11

Val d'Adige Nord	*Renetta canada*	2
	Golden delicious	5
Val d'Adige Sud	*Renetta canada*	1
	Golden delicious	8
	Braeburn	1
Valsugana	*Renetta canada*	5
	Golden delicious	11
	Florina	2
Val del Sarca	*Renetta canada*	1
	Golden delicious	6
	Jonagold	2
	Florina	1
	Red delicius	1
	Granny	1
Bleggio area	*Golden delicious*	9
	Jonagold	1
TOTAL		179

2.1.2 - Second collection

In the second year the collection diagram was modified. Besides the 10 areas considered in the first collection, other 5 micro-areas were inspected for a more intensive analysis. Every micro-area is located respectively in an area of intensive cultivation (Val di Sole and Val di Non areas). The exact location of respective micro-areas was established according to results of strain distribution of the first collection.

In the areas (**Tab. 5**) and in the micro-areas (**Tab. 6**) respectively 198 and 100 samples were collected.

Tab. 5: plant samples collected in 2004.

AREA	CULTIVAR	N° SAMPLES
Val di Sole	*Renetta canada*	7
	Golden delicious	11
	Gala	1
Revò - Alta Val di Non	*Renetta canada*	10
	Golden delicious	10
Tuenno - Cles	*Renetta canada*	10
	Golden delicious	10
Taio - Segno	*Renetta canada*	5
	Golden delicious	15
Denno - Cuneo - Campodenno	*Renetta canada*	10
	Golden delicious	10
Val d'Adige Nord	*Renetta canada*	2
	Golden delicious	7
	Red delicious	3
	Gala	2
	Granny	2
	Stayman	1

Val d'Adige Nord	*Braeburn*	1
	Idared	1
	Morgenduft	1
Val d'Adige Sud	*Renetta canada*	1
	Golden delicious	10
	Red delicius	1
	Gala	1
	Granny	1
	Stayman	1
	Braeburn	1
	Morgenduft	1
	Fuji	1
	Pink lady	1
	Red chief	1
Valsugana	*Renetta canada*	2
	Golden delicious	10
	Red delicious	3
	Gala	2
	Florina	1
	Red chief	1
	Morgenduft	1
Val del Sarca	*Golden delicious*	9
	Red delicious	6
	Gala	3
	Morgenduft	1
Bleggio area	*Golden delicious*	19
	Red delicious	1
TOTAL		198

Tab. 6: micro-area plant samples collected in 2004.

MICRO-AREA	AREA	CULTIVAR	N° SAMPLES
FOCUS Sole - Cavizzana	**Val di Sole**	*Renetta canada*	1
		Golden delicious	18
		?	1
FOCUS Non - Sporminore	**Denno - Cuneo - Campodenno**	*Golden delicious*	20
FOCUS Non - Tuenno	**Tuenno - Cles**	*Renetta canada*	5
		Golden delicious	15
BONIFICA - Rumo	**Revò - Alta Val di Non**	*Golden delicious*	14
		Lasa	4
		Red delicious	1
		Bella di Boskoop	1
BONIFICA - Vervò	**Taio - Segno**	*Renetta canada*	2
		Golden delicious	8
		Fuji	5
		Gala	3
		Red delicious spur	1
		Sansa	1
TOTAL			100

2.1.3 - Third collection

In the third year the collection was conducted in two model regions. The one was situated at Nanno, in Tuenno - Cles area (in the middle of Val di Non). The other was placed in Piovi, in Val d'Adige Nord area.

In these regions 366 plant samples were collected (**Tab. 7**).

Tab. 7: plant samples collected in 2005.

MODEL REGION	AREA	CULTIVAR	N° SAMPLES
Nanno	**Tuenno - Cles**	*Renetta canada*	145
		Golden delicious	58
		Red delicious	6
		?	2
Piovi	**Val d'Adige Nord**	*Golden delicious*	62
		Gala	57
		Red delicious	16
		Braeburn	11
		Granny	4
		Fuji	3
		Morgenduft	2
TOTAL			366

2.2 - PSYLLID SAMPLE COLLECTION

Psyllid captures were carried out twice a week in the apple orchards from late January to early September each year. Samples were taken from several different sites in the upper, medium and lower valley of Val di Non and Val di Sole as well as in the other areas of Trentino. Psyllids were captured with the frappage method (50 branches per date and site). In the first and second year the samples were composed of 10 insects on an average while in the third collection, nearly all the analyses were conducted on a single insect.

C. picta and *C. melanoneura* are the species more widespread. The first is present particularly in Val di Sole and Val di Non while the second is distributed in the rest of Trentino.

Insects split up by the following variables:

- area of origin of the sample,
- species,
- developmental stages.

2.2.1 - First collection

In the first year 981 psyllid samples were captured from all the areas (**Tab. 8**).

Tab. 8: psyllid samples captured in 2002 (Total insects picked up = 8227).

AREA	SPECIES	N° SAMPLES
Val di Sole	*C. picta*	20
	C. melanoneura	38
	C. mali	18
	C. pyri	2
Revò - Alta Val di Non	*C. picta*	137
	C. melanoneura	58
	C. mali	11
	C. pyri	4
	C. pruni	2
	Other species	10
Tuenno - Cles	*C. picta*	95
	C. melanoneura	82
	C. pyri	2
	Trioza	1
	Other species	3
Taio - Segno	*C. melanoneura*	19
Denno - Cunevo - Campodenno	*C. picta*	78
	C. melanoneura	42
	C. pruni	1
Val d'Adige Nord	*C. picta*	12
	C. melanoneura	251
Val d'Adige Sud	*C. picta*	4
	C. melanoneura	24
	C. pruni	1
	Trioza	1
Valsugana	*C. melanoneura*	37
	C. pruni	1
Val del Sarca	*C. picta*	1
	C. melanoneura	18
	C. pruni	1
	Other species	1
Bleggio area	*C. melanoneura*	6
TOTAL		981

2.2.2 - Second collection

In the second year the psyllids were captured only in three areas of Val di Non, in Val d'Adige Nord and in Valsugana areas.

In these areas 974 samples were collected (**Tab. 9**).

Tab. 9: psyllid samples captured in 2004 (Total insects picked up = 3423).

AREA	SPECIES	N° SAMPLES
Revò - Alta Val di Non	*C. picta*	467
	C. melanoneura	30
	C. breviantennata	1
Tuenno - Cles	*C. picta*	85
	C. melanoneura	26
Denno - Cunevo - Campodenno	*C. picta*	10
	C. melanoneura	8
Val d'Adige Nord	*C. picta*	29
	C. melanoneura	314
	C. breviantennata	1
Valsugana	*C. picta*	3
TOTAL		974

2.2.3 - Third collection

In the third year the collection was conducted only in the Nanno and the Piovi model region. In these region 149 psyllid samples were captured (**Tab. 10**).

Tab. 10: psyllid samples captured in 2005 (Total insects picked up = 149).

MODEL REGION	AREA	SPECIES	N° SAMPLES
Nanno	**Tuenno - Cles**	*C. picta*	82
Piovi	**Val d'Adige Nord**	*C. picta*	1
		C. melanoneura	66
TOTAL			149

2.3 - APHID SAMPLE COLLECTION

Aphids were picked up periodically from colonies on plants showing the symptoms of the disease in orchards located in two different valleys of Trentino, Val di Non and Val d'Adige, from early April to early September. All the analyses were conducted on a single insect.
Insects split up by species variable. The presence of phytoplasma was monitored in the five species of apple aphids from 2003 to 2005. The more widespread species are *D. plantaginea* and *A. pomi*.
In 2006 the aphids were captured only in Denno - Cunevo - Campodenno area to establish the presence and the quantity of phytoplasma in different parts of the insect (head and body).

2.3.1 - 2003 collection

In the first year 193 aphid samples were captured in three areas of Val di Non and in Val d'Adige Nord area (**Tab. 11**).

Tab. 11: aphid samples captured in 2003.

AREA	SPECIES	N° SAMPLES
Tuenno - Cles	*Dysaphis plantaginea*	6
	Aphis pomi	15
Taio - Segno	*Dysaphis plantaginea*	45
Denno - Cunevo - Campodenno	*Dysaphis plantaginea*	21
	Aphis pomi	36
Val d'Adige Nord	*Aphis pomi*	70
TOTAL		193

2.3.2 - 2004 collection

In the second year the aphids were captured only in two areas of Val di Non and in Val d'Adige Nord area. In these areas only 133 samples were collected (**Tab. 12**).

Tab. 12: aphid samples captured in 2004.

AREA	SPECIES	N° SAMPLES
Revò - Alta Val di Non	*Dysaphis plantaginea*	15
	Aphis pomi	50
Denno - Cunevo - Campodenno	*Dysaphis devecta*	11
Val d'Adige Nord	*Dysaphis plantaginea*	30
	Aphis pomi	15
	Dysaphis devecta	8
	Rhopalosiphum insertum	4
TOTAL		133

2.3.3 - 2005 collection

In 2005 the aphids were captured only in three areas of Val di Non.

In this year 655 samples were collected (**Tab. 13**).

Tab. 13: aphid samples captured in 2005.

AREA	SPECIES	N° SAMPLES
Revò - Alta Val di Non	*Dysaphis plantaginea*	71
	Aphis pomi	10
Tuenno - Cles	*Dysaphis plantaginea*	205
	Aphis pomi	256
	Eriosoma lanigerum	22
Denno - Cunevo - Campodenno	*Dysaphis plantaginea*	71
	Aphis pomi	10
	Eriosoma lanigerum	10
TOTAL		655

2.3.4 - 2006 collection

In 2006 only 42 aphids were captured in the Denno - Cunevo - Campodenno area to establish the presence and the quantity of phytoplasma in different parts of the insect, separately (head and body). Furthermore four AP positive *C. picta* psyllids were employed as point of reference (**Tab. 14**).

Tab. 14: aphids and psyllids captured in 2006.

INSECT	SPECIES	N° SAMPLES
Aphid	*Dysaphis plantaginea*	26
	Eriosoma lanigerum	16
Psyllid	*C. picta*	4
TOTAL		46

2.4 - SAMPLE LYOPHILIZING

Insect samples and fresh main nervation of leaves (approximately 0,1 g) of each plant sample were placed into a 2 ml eppendorf tube. With the caps open, the tubes were closed with pierced parafilm. In this way samples can't go out but air and liquid nitrogen can go through the parafilm plug. The samples were frozen in liquid nitrogen and stored at - 80 °C until lyophilizing.

Frozen samples were lyophilized in a vacuum pump in approximately 36 hours. It's important that the material is dry otherwise grinding samples with MM300 mixer mill (Retsch,

Germany- **Fig. 27**) can lead to clumps. After the parafilm was removed from each microtube and closed caps. Samples were stored until extractions.

Fig. 27: MM300 mixer mill used to pulverize the samples.

2.5 - DISRUPTION OF SAMPLES

The MM300 mixer mill permits rapid and efficient tissue disruption of 2 x 24 samples in parallel by oscillating a 3 mm tungsten carbide bead in each 2 ml eppendorf tube, together with the sample material. Disruption was performed in two or three 1 minute high-speed (30 Hz) shaking steps. By using lyophilized tissue with MM300 mixer mill, sample disruption was facilitated, improving the final yield.

A 3 mm tungsten carbide bead was placed into the 2 ml eppendorf tube, together with the lyophilized sample material. The tubes were frozen in liquid nitrogen for 30 s and placed into the Mixer Mill Adapter Set 2 x 24. After the fixing of the MM300 mixer mill clamps, the samples were immediately ground for 1 min at 30 Hz. The tubes were removed and re-frozen in liquid nitrogen for 2 minutes.

Last two steps were repeated until all the samples were ground into a fine powder (typically two or three times), reversing the position of tubes within the Adapter Set (in this way all the samples were thoroughly and equally disrupted).

2.6 - DNA EXTRACTION

Extraction of DNA from the main nervation of leaves or insects was done with a CTAB extraction buffer consisting of 3% w/v CTAB (cetyltrimethylammonium bromide), 1,4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid), 1,0 M Tris-HCl (tris-hydroxymethyl-methylamine hydrochloride), 0,2% v/v 2-mercaptoethanol and 1% w/v PVP (Polyvinylpyrollidine) (buffer pH = 8,0) (Doyle and Doyle, 1990).

This buffer (800 µl) was added to the frozen powder.

The tubes were capped and the contents gently mixed. The suspension was incubated for a further 30 minutes at 60 °C and the mixture occasionally swirled.

After incubation, the solutions were extracted once with an equal volume of IAC (chloroform : isoamyl alcohol 24:1 v/v), by mixing gently but thoroughly for about 10 minutes, followed by centrifugation at 10000 x *g* for 15 minutes at room temperature. The upper aqueous phase was transferred to a clean eppendorf tube using a pipette. A 2/3 volume of cold isopropanol (0 °C) was added and the solution was mixed gently to precipitate the DNA.

After centrifugation at 12000 x *g* for 10 minutes at 4 °C, the supernatant was eliminated and the precipitated strands of DNA were washed by 600 µl wash-buffer (76% v/v EtOH, 10 mM NH4Ac). The DNA was washed gently until white. The wash-buffer was discarded after further centrifugation at 12000 x g for 10 minutes at room temperature and the DNA was dried brief1y under extractor hood. The DNA was dissolved in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

Contaminating RNA, which can interfere with DNA quantification, was removed by digestion with RNase A (10 µg/ml final concentration) for 30 minutes at 37 °C. The sample was then diluted with 2 volumes of TE (pH 7.4), 1 ml 7.5 *M* NH4Ac and 2 volumes of cold absolute ethanol (-20 °C). The solution was mixed gently to precipitate the DNA. After centrifugation at 12000 x *g* for 10 minutes at 4 °C the supernatant was eliminated. The precipitated DNA was air-dried and re-dissolved in 100 µl TE (pH 7.4).

2.7 - PCR AMPLIFICATION

PCR amplification of 16S ribosomal DNA spacer region and non-ribosomal DNA was employed for detection of the *Ca.* Phytoplasma mali.

The fAT-rAS primer pair generated from 16S ribosomal DNA spacer region sequence amplifies a DNA fragment approximately 500 bp long from all phytoplasmas of Apple Proliferation group except European Stone Fruit Yellows (Smart *et al.*, 1996).

The AP5-AP4 primer pair derived from the sequence of a 1,8 kbp chromosomal DNA fragment of the *Ca.* Phytoplasma mali and amplifies a fragment 483 bp long (Jarausch *et al.*, 1994).

The sequences of primers are as follows:

primer fAT 5'- CAT CAT TTA GTT GGG CAC TT -3'

primer rAS 5'- GGC CCC GGA CCA TTA TTT ATT -3'

primer AP5 5'- TCT TTT AAT CTT CAA CCA TGG C -3'

primer AP4 5'- CCA ATG TGT GAA ATC TGT AG -3'

The PCR assays were performed with 100-150 ng DNA, 200 µM each dNTP, 0,375 µM each primer, 10x NH_4 Reaction Buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8,8 at 25 °C, 0,1% Tween-20), 1,5 mM $MgCl_2$, 0,5 U Taq polymerase (Bioline, USA) in a final reaction volume of 20 µl.

Thirty-five and 40 PCR cycles were conducted respectively with fAT-rAS and AP5-AP4 primer pair in a GeneAmp® PCR System 9700 (Applied Biosystems, USA).

The PCR parameters were as follows: 30 s of denaturation at 95 °C (3 min for the first cycle), 40 s of annealing and 60 s of extension at 72 °C (9 min in final cycle). Annealing Temperature with fAT-rAS and AP5-AP4 primer pair was respectively 60 and 59 °C.

Healthy samples and reagent blanks were included in each experiment as negative controls. DNA from the infected plant was used as positive control.

PCR amplification products (5µl) were analysed by electrophoresis on 1,5% w/v agarose gels. DNA was stained with ethidium bromide and visualized on a UV transiluminator.

2.8 - PCR-RFLP ANALYSIS

PCR-RFLP analysis is a method used to characterize the *Ca.* Phytoplasma mali strain.

PCR amplification was carried out with AP13-AP10 primers pair derived from the sequence of the 1,8 kbp chromosomal DNA fragment of the phytoplasma, like AP5-AP4 primer pair (Jarausch *et al.*, 2000).

The sequences of primers are as follows:

primer AP13 5'- CTA CAG ATT TCA CAC ATT GG -3'

primer AP10 5'- TTT TCA CAA CGT ATT CCG CC -3'

Reaction mixtures of 20 µl contained 100-150 ng total DNA, 200 µM each dNTP, 0,375 µM each primer, 10x NH_4 Reaction Buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8,8 at 25 °C, 0,1% Tween-20), 2,5 mM $MgCl_2$ and 0,5 U Taq polymerase (Bioline, USA).
PCR reactions were of 40 cycles performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA), preceded by a 2 min denaturation step at 95 °C and followed by an elongation step for 7 min at 72 °C. Cycle conditions were as follows: 20 s of denaturation at 95 °C, 60 s of annealing at 59 °C and 60 s of extension at 72 °C.
The AP13-AP10 primer pair amplifies a fragment 776 bp long.
PCR products were digested with BspH I and Hinc II restriction enzymes according to the manufacturer's instructions (New England BioLabs, USA).
The recognition sites of BspH I and Hinc II are respectively the following:

5' - T$^{\downarrow}$CATGA - 3'	5' - GTPy$^{\downarrow}$PuAC - 3'
3' - AGTAC$_{\uparrow}$T - 5'	3' - CAPu$_{\uparrow}$PyTG - 5'

↓↑ = Sites of cleavage; Py = pyrimidine: T or C; Pu = purine: A or G.

BspH I restriction mixture of 20 µl contained 6 µl of PCR product, 10x NE Buffer 4 (500 mM potassium acetate, 200 Mm Tris-acetate, 100 mM magnesium acetate, 10 mM Dithiothreitol, pH 7,9 at 25 °C) and 2 U of restriction enzyme.
Similarly Hinc II restriction mixture of 20 µl contained 6 µl of PCR product, 10x NE Buffer 3 (1 M NaCl, 200 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM Dithiothreitol, pH 7,9 at 25 °C), 0,1 mg/ml BSA (Bovine serum albumin) and 2 U of restriction enzyme.
The mixtures were digested at 37 °C for 16 hours.
Restriction enzyme digests (5 µl) were analysed by agarose gel electrophoresis using a mixture of 1% agarose (Sigma, USA). DNA was stained with ethidium bromide and visualized on a UV transiluminator.

2.9 - CAPILLARY ELECTROPHORESIS (SNaPshot™)

Another analysis used to characterize the *Ca.* Phytoplasma mali strains utilizes the single-base extension method (microsequencing) for SNP detection on an automated DNA sequencer (capillary electrophoresis).

It's an effective solution for the rapid validation and screening of single nucleotide polymorphisms (SNPs).

A schematic diagram of the method for SNP detection is shown in **Figure 28**.

A primer is designed such that its 3′ nucleotide is one nucleotide 5′ of a target SNP. During the reaction, only fluorescent-labelled dideoxynucleotides are available to extend the 3′-end of a primer in a primer–template molecule. Thus, primer extension proceeds for only a single nucleotide before polymerization is halted, and the resulting fragment is fluorescent-labelled for detection on an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyzer).

The fluorescent dyes are assigned to the individual ddNTPs as in **Tab. 15**.

In order to multiplex the microsequencing reaction, primers must have common annealing requirements and differ in length by at least 3–4 bp to avoid overlapping peaks on the chromatogram. To achieve this size disparity, different-length strings of nonannealing bases are added to the 5′-end of the primers (Belfiore *et al*., 2003).

Two primers derived from the reverse DNA strand of the sequence used for PCR-RFLP analysis.

The sequences of primers are as follows:

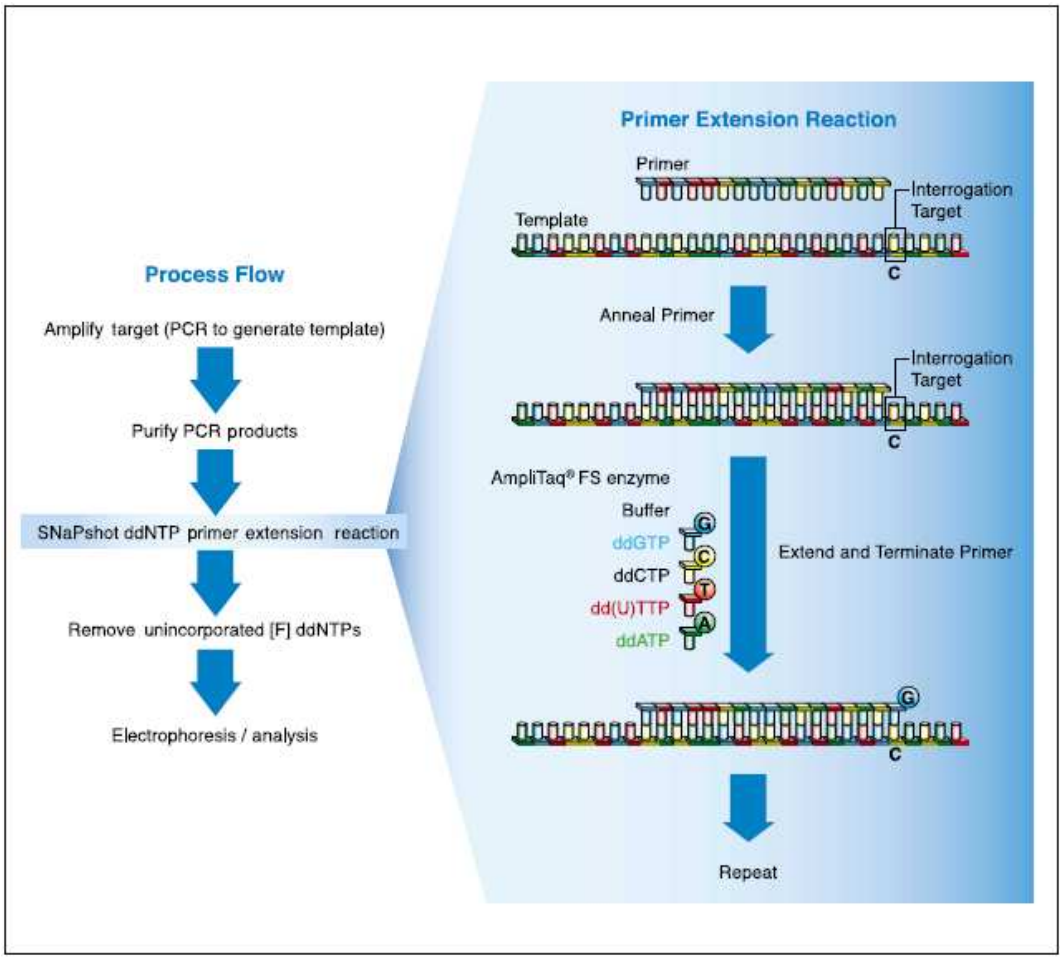
primer snpAPG1r 5'- AGT GCC ATT AAT TCC TTC AT -3'

primer snpAPC2r 5'- TTT TTT TTA ATG TAC TTG TTT ATG TTG T -3'

These primers allow respectively the analysis on the first and second SNP in the sequence. In this case a poly(T) is added to the 5′-end of the primer snpAPC2r.

After PCR amplification with primer AP13-AP10, as in PCR-RFPL analysis, the resulting template is in solution, along with primers, dNTPs, enzyme and buffer components. To avoid participation in the subsequent primer-extension reaction, primers and unincorporated dNTPs must be removed.

Fig. 28: the single base extension approach. The primer anneals adjacent to the target SNP. Labelled ddNTPs in the reaction mix extend a single base, thus terminating the reaction and labelling the product, which is the size of the primer (in this example, 16 bp) plus one nucleotide. ("ABI PRISM® SNaPshot™ Multiplex System" product bulletin genetic analysis - Applied Biosystems, USA).



Tab. 15: the four ddNTPs and their respective fluorescent dyes.

ddNTP	Dye Label	Color of Analyzed Data
A	dR6G	Green
C	dTAMRA™	Black
G	dR110	Blue
T (U)	dROX™	Red

ExoSAP-IT® (Exonuclease I and Shrimp Alkaline Phosphatase in a specially formulated buffer, USB Corporation – USA) allows purification of the template. Two µl of ExoSAP-IT®

were mixed with 5 µl of post-PCR reaction product and incubated at 37 °C for 45' to degrade remaining primers and nucleotides. ExoSAP-IT® was inactivated by following incubation at 75 °C for 15'. This way PCR product is ready for use in DNA sequencing or SNP analyses.
The following SNaPshot™ reaction mix (10 µl) was prepared on ice for each primer: 5,5 µl PCR product (0,01 to 0,40 pmol estimated), 2 µl SNaPshot™ Multiplex Ready Reaction Mix (AmpliTaq® DNA Polymerase FS, Fluorescently labeled ddNTPs, Reaction buffer) (Applied Biosystems,USA) and 0,9 pmol primer.
SNaPshot™ PCR reactions were of 25 cycles performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA), preceded by a 1 min denaturation step at 96 °C. Cycle conditions were as follows: 10 s of denaturation at 96 °C, 5 s of annealing at 50 °C and 30 s of extension at 60 °C.
Then the resulting products were separated electrophoretically in a single capillary on the ABI PRISM® 3100 Genetic Analyzer in the presence of a fifth-dye-labeled internal size standard (GeneScan™-120 LIZ™ size standard, it is specifically designed for small fragments to analyze). Formamide (9,45 µl) was added to 0,5 µl of SNaPshot™ products and 0,05 µl of GeneScan™-120 LIZ™ size standard into each tube.

2.10 - QUANTITATIVE PCR

Phytoplasma load in plant and insect samples was detected by q-PCR based on a TaqMan assay (Baric *et al.*, 2004).
It was possible to establish the absolute and relative quantity of phytoplasma in an insect (n° copies/insect) and in the main nervation of leaves (n° copies / pg DNA) respectively.
The cloning of a 70bp phytoplasma DNA fragment in a plasmid vector allowed the construction of phytoplasma Standard Curve necessary for pathogen quantitative analysis.
The sequence of this fragment, localized in 16S rRNA gene, was deposited in GenBank under accession number AY 510067 (Baric *et al.*, 2004).
The sequence of the fragment is as follows:

1 5'- CATTGTTGTC GATATTAACA TGTATAATGG GACTCTATCT TTATTCTCGT
51 CCGATTAATC AATTATTAAA AAGATCTATC AGACTACGGT GGAGTGAATG
101 GTTTGATCAA TAAATATTCG ATTCTTTTTT CAATTTTTAA TCGATTCACA
151 ACAAGTCTTT CATTTTTCAT ATAAATATAA AAAAATACAG ATTTGGGTCG

201 TCATTAATCA TTTTGAGATA GTATTTCAGT ACTATACGTA TGTATATAGG
250 TTTATCCTTC ATCCTTTCTG AAGTTTCGAT GGAAGGATTC CTTTACTAAC
301 ACAATGCAGC CAACTCCATT TGTTAGAACA GCTTCCATTG AGTCTCTGCA
351 CCTATCCTTT TTTATTTTCG GTTTATGAAA CCCTTGTTTA TTTTCATAAA
401 ACAGGATTTG GCTCAGGATT GCCCATTTTT AATTCCAGGG TTTCTCTGAA
451 TTTGAAAGTT CTCACTTGGT AGGTTTCC – 3'

This ribosomal gene is present in two copies in the phytoplasma genome (Schneider and Seemüller, 1994), thus increasing the sensitivity of a diagnostic assay. This fact might be important in situations where pathogen titer in plant material is low.

Primers and probes for detection of *Ca.* Phytoplasma mali were matched sequence in a variable region of the 16S rRNA gene starting at position 82 and ending at position 157.

A second primer/probe combination was designed as an internal positive control for multiplex real-time PCR. The slowly evolving chloroplast gene of *M. domestica* coding for tRNA leucine was selected to assure amplification of host 69 bp DNA fragment even when diverse apple cultivars or different *Malus* species are surveyed (Taberlet *et al*., 1991).

The probes and primers for a simultaneous detection of AP phytoplasma and its host plant in a realtime PCR assay were obtained from Applied Biosystems (**Tab. 16**). The probe for detecting AP phytoplasma was conjugated with the reporter dye FAM at its 5' end while the probe for apple chloroplast DNA amplification was conjugated with the reporter dye VIC. Both probes were attached with a Minor Groove Binder (MGB) and a non-fluorescent quencher dye (NFQ) at their 3' ends (Applied Biosystems, USA).

Tab. 16: primers and probes used in quantitative PCR.

Name	Specificity	Sequence 5' → 3'
qAP-16S-F	AP phytoplasma	CGA ACG GGT GAG TAA CAC GTA A
qAP-16S-R	AP phytoplasma	CCA GTC TTA GCA GTC GTT TCC A
qAP-16S	AP phytoplasma	**FAM**-TAA CCT GCC TCT TAG ACG
qMd-cpLeu-F	M. domestica	CCT TCA TCC TTT CTG AAG TTT CG
qMd-cpLeu-R	M. domestica	AAC AAA TGG AGT TGG CTG CAT
qMd-cpLeu	M. domestica	**VIC**-TGG AAG GAT TCC TTT ACT AAC

For each target, the optimal primer concentrations were determined in separate tubes by running a matrix of forward and reverse primer concentrations according to Applied Biosystems user bulletin no. 5 (P/N 4306236B; http://www.appliedbiosystems.com).

In a subsequent multiplex optimization experiment, the primer concentration of qMd-cpLeu was limited while the concentration of both qAP-16S primers was kept constant at 900 nM. The adjustment of primer concentrations in the multiplex real-time PCR assay was necessary to avoid competition between the two systems and obtain two independent reactions in a single tube. Since chloroplast DNA was expected to be more abundant in total DNA extracts, amplification of chloroplast DNA was assumed to use up the common reagents and impair amplification of phytoplasmal DNA. Therefore, primer concentrations of the internal positive control were limited so that primers would be exhausted soon after obtaining CT values. In this way, sufficient amounts of reagents would be available to amplify pathogen DNA and ensure reliable diagnosis.

Multiplex reactions were carried out in a total volume of 20 µl containing 10–100 ng of template DNA, 2x TaqMan Universal PCR Master Mix® (Applied Biosystems, USA), 900 nM of both qAP-16S primers, 100 nM of both qMd-cpLeu primers and 200 nM of each probe. Amplification and detection were performed using an automated ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, USA) in MicroAmp® optical 96-well plates. PCR was initiated with two incubation steps: 2 min at 50 °C to activate AmpErase® UNG (Applied Biosystems, USA), which prevents reamplification of carry-over PCR products, 10 min at 95 °C to activate AmpliTaq Gold® DNA polymerase (Applied Biosystems, USA), followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each reaction included a negative control without template.

The phytoplasmal DNA Standard Curve used to quantitative analysis of plant and insect samples was prepared with threefold serial dilutions ranging from an average of 3 millions to 4000 phytoplasma copy.

The plant DNA Standard Curve used only for plant quantitative analysis was prepared with DNA of well-known concentration of a *Golden delicious* healthy sample. The fivefold serial dilutions ranged from an average of 65000 to 100 pg of DNA.

Threshold levels were set to intersect the amplification curves in the linear region of the semilog plot. Threshold cycles for each PCR reaction were calculated with ABI Prism® 7000 SDS Software (version 1.1).

2.11 - CLONING OF PHYTOPLASMA FRAGMENT DNA

Quantitative PCR analysis requires a Standard Curve to establish the exact quantity of phytoplasma in the sample. A pathogen DNA fragment within a plasmid is the best system to have a correct Standard Curve. TOPO® XL PCR Cloning Kit (Invitrogen, USA) was used to clone the DNA fragment in plasmid.

The map of **Figure 29** shows the features of pCR®-XL-TOPO® and the sequence surrounding the TOPO TA Cloning® site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. The complete sequence of the vector is available for downloading from Invitrogen web site (www.invitrogen.com).

Naturally the same sequence used for quantitative PCR was amplified in the same conditions and protocol with primers qAP-16S-F and qAP-16S-R in a GeneAmp® PCR System 9700 (Applied Biosystems, USA).

Later 5 µl from each PCR amplification were removed and analyzed by 1,5% agarose gel electrophoresis. This was to ensure production of a single discrete band of the correct size.

Quantification of DNA was done by spectrophotometry at 260/280 nm (GeneQuant pro RNA/DNA Calculator - Biochrom, UK).

2.11.1 - TOPO® Cloning reaction

Cloning was done on 1:10 dilution of PCR products because it was too concentrated (470 ng/µl).

The 5 µl TOPO® Cloning reaction (10 copies) was set up adding 4 µl PCR product and 1 µl pCR®-XL-TOPO® vector (10 ng/µl plasmid DNA in: 50% glycerol, 50 mM Tris-HCl, pH 7,4 at 25 °C, 1 mM EDTA, 2 mM DTT, 0,1% Triton X-100, 100 µg/ml BSA, phenol red) in every sterile microcentrifuge tube.

It was mixed gently and incubated for 5 minutes at room temperature (~25 °C).

Later 1 µl 6x TOPO® Cloning Stop Solution (0,3 M NaCl, 0,06 M MgCl2) was added and was mixed for several seconds at room temperature. Addition of the Stop Solution increases the yield of transformants by an average of 2-fold.

The tubes were briefly centrifuged and placed on ice.

2.11.2 - Transformation by chemically competent cells

Every TOPO® Cloning reaction (2 µl) was added in one vial of One Shot® cells and mixed gently avoiding pipetting up and down.

Later these were incubated on ice for 30 minutes.

The cells were heat-shocked for 30 seconds at 42 °C without shaking and immediately incubated on ice for 2 minutes.

Later 250 µl room temperature S.O.C. medium (2% Tryptone, 0,5% Yeast Extract, 10 mM NaCl, 2,5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was added.

The tubes were capped tightly and shook horizontally at 37 °C for 1 hour.

Afterwards they were place on ice.

Two hundred µl of each transformation were spread on a prewarmed plate and incubated overnight at 37 °C.

2.11.2.1 - Preparation of LB (Luria-Bertani) medium

In 1 litre of deionized water, 10 g tryptone, 5 g yeast extract and 10 g NaCl was dissolved.

The pH of the solution was adjusted to 7,0 with NaOH.

It was autoclaved on liquid cycle for 20 minutes at 1 atm.

When the solution was cooled to 55 °C kanamycin antibiotic (50 µg/ml) was added.

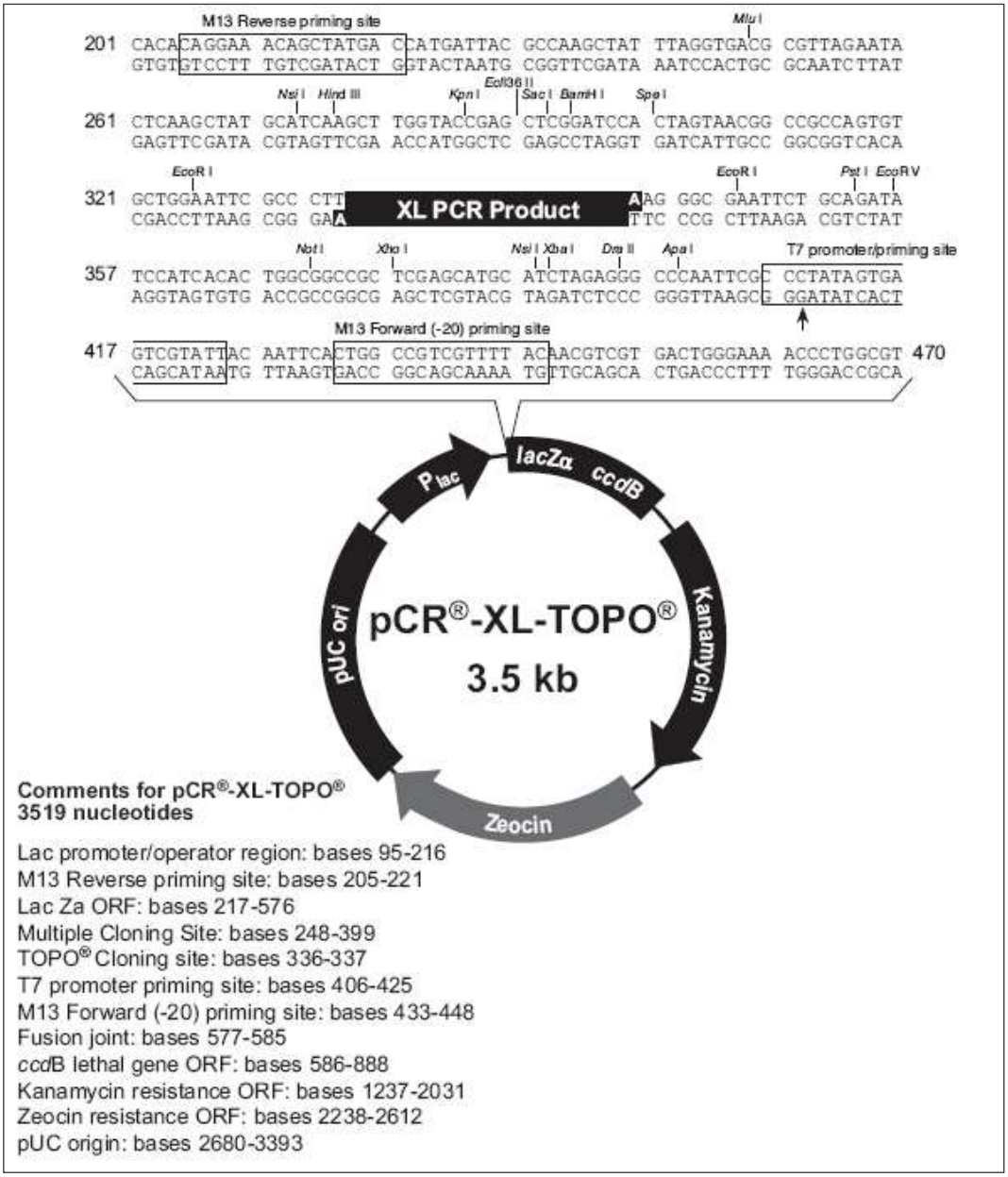
2.11.2.2 - Preparation of LB agar plates

The same protocol for the preparation of LB medium was used but 15g agar was added before autoclaving.

After autoclaving, when the solution was cooled to 55 °C, kanamycin antibiotic (50 µg/ml) was added.

The preparation was poured into 10 cm plates.

Fig. 29: the sequence of TOPO TA Cloning® site ("TOPO® XL PCR Cloning Kit" Instruction Manual - Invitrogen, USA).



2.12 - ISOLATION OF PLASMID DNA

Sequencing is necessary for the analysis of positive clones. Therefore plasmid DNA was necessary isolated from transforming cells.

Ten colonies were picked and were grown overnight in LB medium containing 50 µg/ml kanamycin. These represent the stocks of possible positive clones and are maintained in glycerol (Sigma, USA) at - 80 °C.

Afterwards the tubes containing colonies and LB medium were centrifuged at 4500 x *g* for 5 minutes at 4 °C.

The supernatants were eliminated and the pellets were dissolved in 1,5 ml Plasmid Prep 1 (50 mM Glucose, 10mM EDTA, 25mM Tris-HCl pH 8) and 3 mg/ml lysozyme (Sigma, USA).

They were incubated on ice for 30 minutes.

Two ml Plasmid Prep 2, consisting of 0,2 N NaOH and 1% SDS (sodium dodecyl sulfate), was added and mixed gently and the mixture was incubated on ice for 5 minutes.

Plasmid Prep 3 (2 M sodium acetate ph 4,8) (2 ml) was added and mixed gently.

The tubes were incubated on ice for 1 hour and centrifuged at 4500 x *g* for 10 minutes at room temperature.

The supernatant were transferred in clean tubes with 1 µl RNAse A (10 µg/ml final concentration) and incubated for 30 minutes at 37 °C.

A volume of phenol and IAC was added in every tube and centrifuged at 12000 x *g* for 5 minutes.

The supernatant were transferred in clean tubes with a volume of IAC and centrifuged at 12000 x *g* for 5 minutes.

Again the supernatant were transferred to clean tubes and two volumes of ethanol were added.

The solutions were mixed gently to precipitate the plasmid DNA and they were incubated for 30 minutes at - 80 °C.

After centrifugation at 12000 x *g* for 20 minutes at 4 °C, the supernatant were eliminated and the precipitated were washed by 600 µl 70% v/v EtOH.

The ethanol was discarded after further centrifugation at 12000 x g for 10 minutes at room temperature and every plasmid DNA sample was dried brief1y under extractor hood.

These were dissolved in 100 µl water and 140 µl 5M NaCl and PEG 13% (polyethylene glycol) solution (Sigma, USA) (1:1 v/v) was added.

The mixture was incubated on ice for 10 minutes and centrifuged at 12000 x *g* for 10 minutes at 4 °C.

The supernatants were eliminated and the precipitated plasmid DNA were washed by 300 µl 70% v/v EtOH.

The ethanol were discarded after further centrifugation at 12000 x g for 10 minutes at room temperature and every plasmid DNA sample was dried brief1y under extractor hood.

Finally these plasmid DNA were dissolved in 40 µl water and quantified by spectrophotometry at 260/280 nm (GeneQuant pro RNA/DNA Calculator - Biochrom, UK).

2.13 - SEQUENCING

The analysis of plasmids by sequencing required two DNA amplifications by PCR with M13 Forward and M13 Reverse primers separately.

The sequences of primers are as follows:

primer M13 Forward 5'- GTA AAA CGA CGG CCA G -3'

primer M13 Reverse 5'- CAG GAA ACA GCT ATG AC -3'

Reaction mixtures of 20 µl contained 1,5 µl plasmid DNA (it's depend on DNA fragment length and concentration), 5x Big Dye Terminator® Sequencing Buffer, 4 µl Big Dye Terminator® v3.1 and 3,2 pmol primer (Big Dye Terminator® v3.1 Cycle Sequencing Kit - Applied Biosystems, USA).

PCR reactions were of 30 cycles performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA), preceded by a 5 min denaturation step at 95 °C and followed by an elongation step for 7 min at 72 °C. Cycle conditions were as follows: 30 s of denaturation at 95 °C, 10 s of annealing at 55 °C and 240 s of extension at 60 °C.

Afterwards the plasmid DNA amplification was purified by columns of Sephadex G50-fine (Amersham Biosciences, UK).

Formamide (15 µl) is added to 5 µl of purified products in each tube and sequenced by ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

The sequencing confirmed that the phytoplasma DNA fragment was cloned in the correct orientation in two samples of plasmid DNA.

3 - RESULTS AND DISCUSSIONS

3A - POPULATION STRUCTURE OF AP IN TRENTINO

3A.1 - PHYTOPLASMA DETECTION

All samples were subjected to PCR analysis with fAT-rAS and AP5-AP4 primer pair for the detection of *Ca.* Phytoplasma mali (**Fig. 30**).

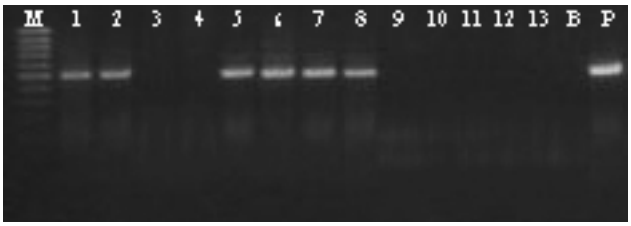
The PCR results of plant and insect samples were shown year by year in succession.

Fig. 30: examples of the results of phytoplasma detection. PCR amplification products were analysed by electrophoresis on 1,5% w/v agarose gels. DNA was stained with ethidium bromide and visualized on a UV transiluminator. M = Marker (1000 bp); B = Blank; P = positive control.

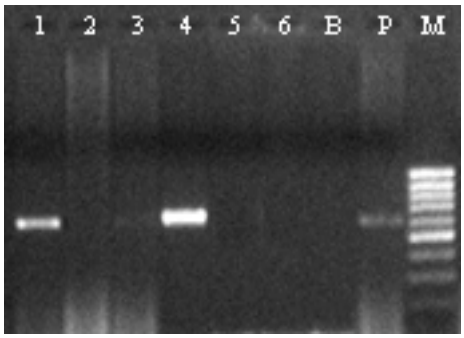
A - PCR with fAT-rAS primer pair; 1, 2, 5-8 = positive samples; 3, 4, 9-13 = negative samples.

B - PCR with AP5-AP4 primer pair; 1, 4 = positive samples; 2, 3, 5, 6 = negative samples.

A



B



3A.1.1 - Plant samples

In three years 843 leave samples were collected from apple trees that showed the symptoms of the disease. Evidently nearly all the samples collected in three years were AP positive (797 out of 843 total plant samples) since they were collected from apple trees that showed the typical symptoms of the disease.

3A.1.1.1 - First collection

In the first year 169 out of 179 plant samples were AP positive (**Tab. 17**). Not all generated the PCR fragment with primers AP5/AP4 even if they were positive to the test with primers fAT/rAS. *Golden delicious* samples were present in all the areas while those of *Renetta del Canada* were frequent only in Val di Sole and in four Val di Non areas.

Tab. 17: analysis of the plant samples collected in 2002.

AREA	CULTIVAR	N° SAMPLES	N° SAMPLES INFECTED
Val di Sole	*Renetta canada*	14	14
	Golden delicious	14	12
	Florina	2	2
Revò - Alta Val di Non	*Renetta canada*	12	11
	Golden delicious	14	13
	Gala	1	1
Tuenno - Cles	*Renetta canada*	15	15
	Golden delicious	10	10
	Florina	1	0
Taio - Segno	*Renetta canada*	10	10
	Golden delicious	10	10
Denno - Cunevo - Campodenno	*Renetta canada*	8	8
	Golden delicious	11	10
Val d'Adige Nord	*Renetta canada*	2	2
	Golden delicious	5	4
Val d'Adige Sud	*Renetta canada*	1	1
	Golden delicious	8	7
	Braeburn	1	1
Valsugana	*Renetta canada*	5	5
	Golden delicious	11	11
	Florina	2	2
Val del Sarca	*Renetta canada*	1	1
	Golden delicious	6	6
	Jonagold	2	2
	Florina	1	1
	Red delicius	1	1
	Granny	1	0
Bleggio area	*Golden delicious*	9	8
	Jonagold	1	1
TOTAL		179	169

3A.1.1.2 - Second collection

In the second year 179 out of 198 plant samples collected in the areas were AP positive (**Tab. 18**) while in the micro-areas 93 out of 100 plant samples were AP positive (**Tab. 19**).

Tab. 18: analysis of the plant samples collected in 2004.

AREA	CULTIVAR	N° SAMPLES	N° SAMPLES INFECTED
Val di Sole	*Renetta canada*	7	6
	Golden delicious	11	8
	Gala	1	1
Revò - Alta Val di Non	*Renetta canada*	10	10
	Golden delicious	10	9
Tuenno - Cles	*Renetta canada*	10	10
	Golden delicious	10	9
Taio - Segno	*Renetta canada*	5	5
	Golden delicious	15	14
Denno - Cuneo - Campodenno	*Renetta canada*	10	9
	Golden delicious	10	10
Val d'Adige Nord	*Renetta canada*	2	1
	Golden delicious	7	7
	Red delicious	3	3
	Gala	2	2
	Granny	2	2
	Stayman	1	1
	Braeburn	1	1
	Idared	1	1
	Morgenduft	1	1
Val d'Adige Sud	*Renetta canada*	1	1
	Golden delicious	10	10
	Red delicius	1	1
	Gala	1	0
	Granny	1	1
	Stayman	1	1
	Braeburn	1	1
	Morgenduft	1	1
	Fuji	1	1
	Pink lady	1	1
	Red chief	1	1
Valsugana	*Renetta canada*	2	1
	Golden delicious	10	9
	Red delicious	3	3
	Gala	2	2
	Florina	1	0
	Red chief	1	1
	Morgenduft	1	1
Val del Sarca	*Golden delicious*	9	9
	Red delicious	6	5
	Gala	3	3
	Morgenduft	1	1
Bleggio area	*Golden delicious*	19	14
	Red delicious	1	1
TOTAL		198	179

For this collection *Golden delicious* samples were present in all the areas and micro-areas while *Renetta del Canada* were frequent only in Val di Sole and in four Val di Non areas but not in the micro-areas (8% of the samples).

Tab. 19: analysis of the plant samples collected in the micro-areas in 2004.

MICRO-AREA	CULTIVAR	N° SAMPLES	N° SAMPLES INFECTED
FOCUS Sole - Cavizzana	*Renetta canada*	1	1
	Golden delicious	18	14
	?	1	1
FOCUS Non - Sporminore	*Golden delicious*	20	19
FOCUS Non - Tuenno	*Renetta canada*	5	5
	Golden delicious	15	15
BONIFICA - Rumo	*Golden delicious*	14	14
	Lasa	4	4
	Red delicious	1	1
	Bella di Boskoop	1	0
BONIFICA - Vervò	*Renetta canada*	2	2
	Golden delicious	8	8
	Fuji	5	5
	Gala	3	2
	Red delicious spur	1	1
	Sansa	1	1
TOTAL		100	93

3A.1.1.3 - Third collection

In the third year the collection was conducted in the model region of Nanno (in Tuenno - Cles area) and Piovi (in Val d'Adige Nord area).

In these areas 356 out of 366 plant samples were AP positive (**Tab. 20**). Even for this collection *Golden delicious* samples were present in all the areas. Whereas in Nanno model region *Renetta del Canada* was the major cultivar (69% of the samples), it was fully absent in Piovi where *Gala* was frequent (37%).

Tab. 20: analysis of the plant samples collected in 2005.

AREA	CULTIVAR	N° SAMPLES	N° SAMPLES INFECTED
Nanno model region	*Renetta canada*	145	143
	Golden delicious	58	55
	Red delicious	6	6
	?	2	1
Piovi model region	*Golden delicious*	62	61
	Gala	57	54
	Red delicious	16	16
	Braeburn	11	11
	Granny	4	4
	Fuji	3	3
	Morgenduft	2	2
TOTAL		366	356

3A.1.2 - Psyllid samples

In three years 11799 psyllids were picked up and split up by species and developmental stages. In the first and second collection the most samples were composed of 10 insects on an average while in the third collection, nearly all the analyses were conducted on a single insect. Besides the situation of the plant the psyllid samples showed a lower percentage of infection (only 263 out of 2104 samples were AP positive). But this is evident because it is possible to observe the symptoms of the disease in plants but not in insects.

3A.1.2.1 - First collection

A variable number of insects was collected in all the areas of Trentino. *C. picta* was mainly present in Val di Sole area and in four Val di Non areas. *C. melanoneura* was found in all areas but it was widespread in the areas of Trentino where the apple orchards are not intensively grown and the disease plants are less present.

In the first collection 162 out of 981 psyllid samples were resulted positive by PCR analysis (**Tab. 21**). *C. picta* was found infected more than *C. melanoneura*.

Tab. 21: analysis of the psyllid samples captured in 2002 (Total insects picked up = 8227).

AREA	SPECIES	N° SAMPLES	N° SAMPLES INFECTED
Val di Sole	*C. picta*	20	6
	C. melanoneura	38	3
	C. mali	18	2
	C. pyri	2	0
Revò - Alta Val di Non	*C. picta*	137	41
	C. melanoneura	58	0
	C. mali	11	1
	C. pyri	4	0
	C. pruni	2	0
	Other species	10	0
Tuenno - Cles	*C. picta*	95	28
	C. melanoneura	82	5
	C. pyri	2	0
	Trioza	1	0
	Other species	3	0
Taio - Segno	*C. melanoneura*	19	0
Denno - Cunevo - Campodenno	*C. picta*	78	42
	C. melanoneura	42	2
	C. pruni	1	0
Val d'Adige Nord	*C. picta*	12	3
	C. melanoneura	251	19
Val d'Adige Sud	*C. picta*	4	1
	C. melanoneura	24	5
	C. pruni	1	0
	Trioza	1	0
Valsugana	*C. melanoneura*	37	3
	C. pruni	1	0
Val del Sarca	*C. picta*	1	0
	C. melanoneura	18	1
	C. pruni	1	0
	Other species	1	0
Bleggio area	*C. melanoneura*	6	0
TOTAL		981	162

3A.1.2.2 - Second collection

The insects were collected only in three areas of Val di Non, in Val d'Adige Nord and Valsugana areas. Also for this collection the elevated presence of *C. picta* and C. *melanoneura* respectively in Val di Non and in Val d'Adige was remarkable.

In these areas 100 out of 974 psyllid samples were AP positive by PCR analysis (**Tab. 22**). Also in this year *C. picta* was found infected more than *C. melanoneura* even though for both the species the percentage of the samples infected is reduced considerably.

Tab. 22: analysis of the psyllid samples captured in 2004 (Total insects picked up = 3423).

AREA	SPECIES	N° SAMPLES	N° SAMPLES INFECTED
Revò - Alta Val di Non	*C. picta*	467	52
	C. melanoneura	30	0
	C. breviantennata	1	0
Tuenno - Cles	*C. picta*	85	33
	C. melanoneura	26	4
Denno - Cunevo - Campodenno	*C. picta*	10	2
	C. melanoneura	8	1
Val d'Adige Nord	*C. picta*	29	4
	C. melanoneura	314	4
	C. breviantennata	1	0
Valsugana	*C. picta*	3	0
TOTAL		974	100

3A.1.2.3 - Third collection

In the third year the collection of the plant samples as well as the insect samples was conducted in Nanno and Piovi model regions only. *C. melanoneura* was not present in Nanno and only one specimen of *C. picta* was collected in Piovi. In these areas only 4 out of 149 samples resulted positive by PCR analysis (**Tab. 23**). All infected samples came from Nanno.

Tab. 23: analysis of the psyllid samples captured in 2005 (Total insects picked up = 149).

AREA	SPECIES	N° SAMPLES	N° SAMPLES INFECTED
Nanno model region	*C. picta*	82	4
Piovi model region	*C. picta*	1	0
	C. melanoneura	66	0
TOTAL		149	4

3A.2 - STRAIN ANALYSIS

The PCR positive samples were analysed by PCR-RFLP or SNaPshot™ techniques to establish the kind of *Ca.* Phytoplasma mali strain present (**Fig. 31** - **32**).

The results of plant and insect samples were shown year after year in succession.

Fig. 31: examples of the results of PCR-RFLP analysis. The products were analysed by electrophoresis on 1,5% w/v agarose gels. DNA was stained with ethidium bromide and visualized on a UV transiluminator. M = Marker (1000 bp); 1-4 = PCR products digested with BspH I restriction enzymes; 1*-4* = the same PCR products digested with Hinc II restriction enzymes; 1-1*, 3-3*, 4-4* = three AT2 samples; 2-2* = AP sample.

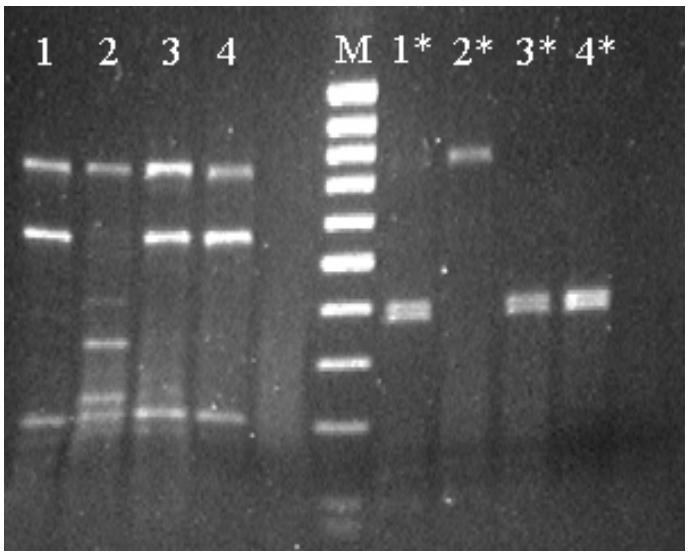
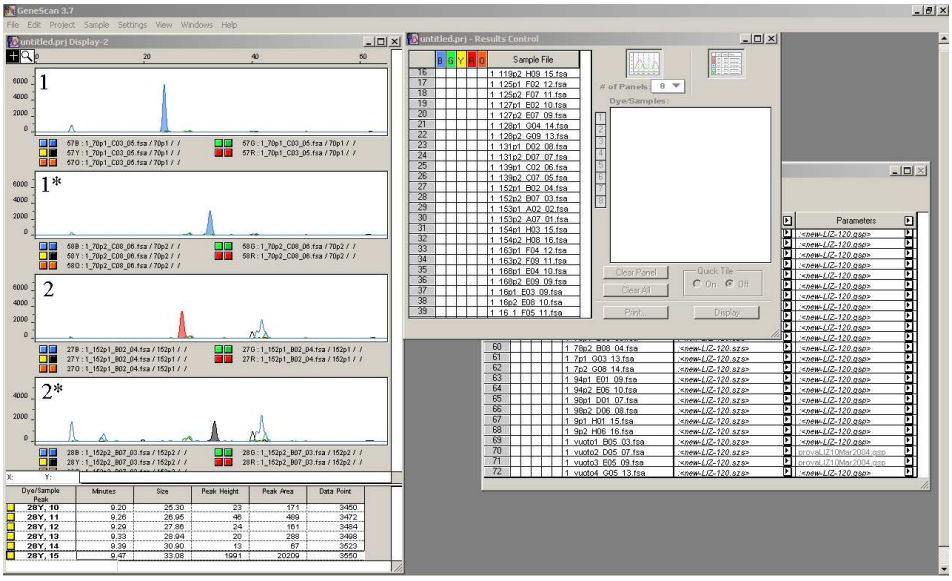


Fig. 32: examples of the results of SNaPshot™ analysis. The PCR products were analysed by capillary electrophoresis with an automated DNA sequencer. 1-1* = AP sample; 2-2* = AT2 sample.



3A.2.1 - Plant samples

Several variables were considered during the strain analysis performed on 797 AP positive samples collected for three years:

- area of origin of the sample,
- cultivar of plant,
- type of rootstock,
- age of plant.

3A.2.1.1 - First collection

The AP positive samples were 169 in the first year, split up in 10 different areas of Trentino.

The data more evident is the high presence of AT2 strain, particularly in the Val di Sole area and the four areas of Val di Non (high infection zone) (**Fig. 33**). Precisely, only the AT2 strain was present in Revò - Alta Val di Non and in the Taio - Cles area. Both the areas are located on the oriental side of Val di Non, on the left bank of the River Noce.

The AT1 strain was spread in Val d'Adige Sud, Valsugana and Bleggio areas, situated in South, South-East and South-West of Trentino, respectively. In the valleys of North-West Trentino the AT1 strain was located only in one orchard of Cavizzana (Val di Sole area, two samples) and one orchard of Sporminore (Denno - Cunevo - Campodenno area, two samples).

AP strain is present only in one sample of Tuenno in Tuenno - Cles area.

The *Renetta del Canada* cultivar present a higher percentage of AT2 strain than *Golden delicious*. This difference is accentuated in the areas of Central and South Trentino (low infection zone) only (**Fig. 34-35**).

The major rootstocks don't present a great difference in the distribution of the strain (**Fig. 36**).

The **Figure 37** shows the distribution of the strains according to the age of the plant samples, in 2002. The data cannot be significant as there are few samples with a great difference in age.

Fig: 33: distribution of the strains in the different areas, in 2002. % AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

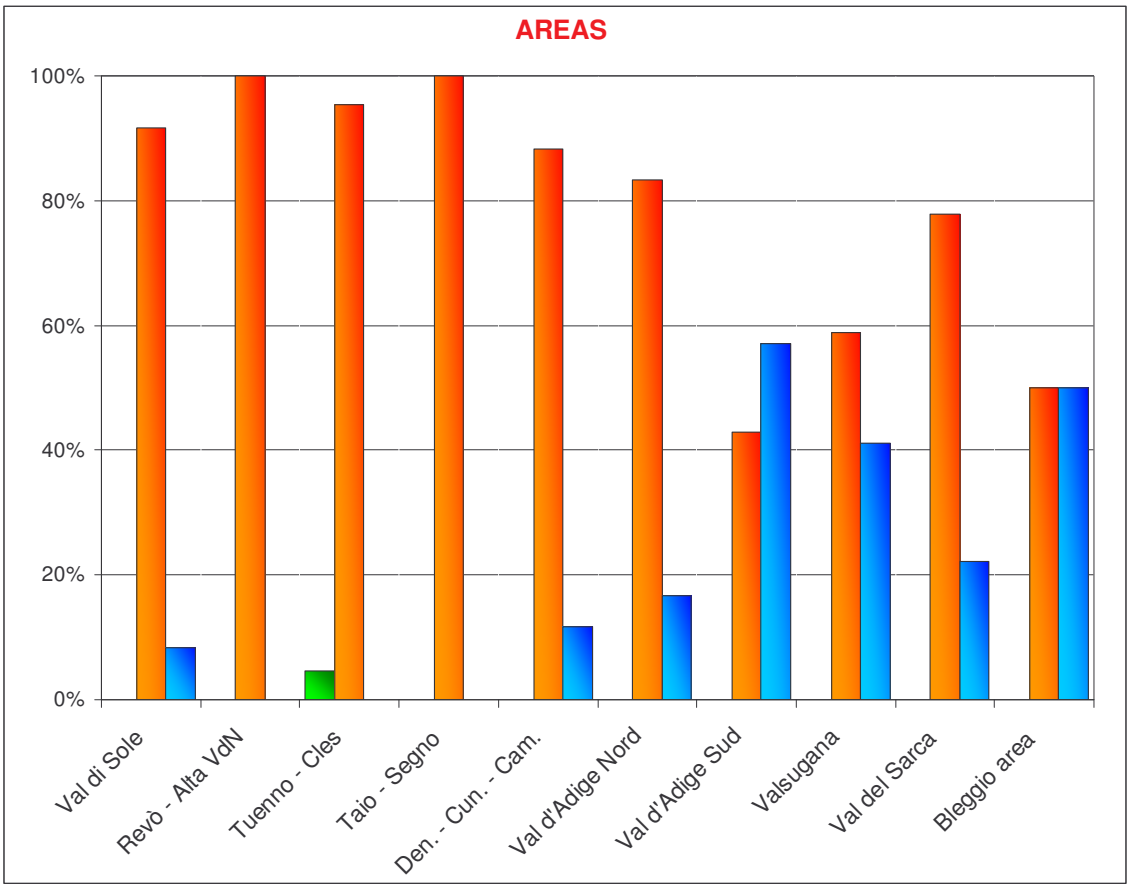


Fig: 34: distribution of the strains in the major cultivars, in the areas of North-West Trentino (high infection zone) in 2002. % AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

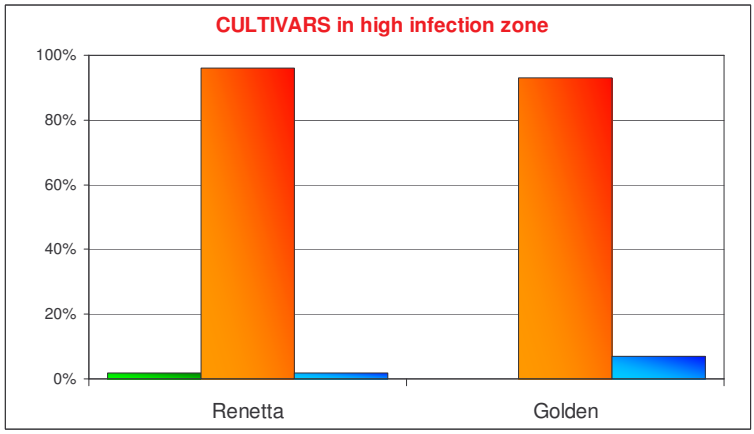


Fig: 35: distribution of the strains in the major cultivars in the areas of Central and South Trentino (low infection zone) in 2002. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

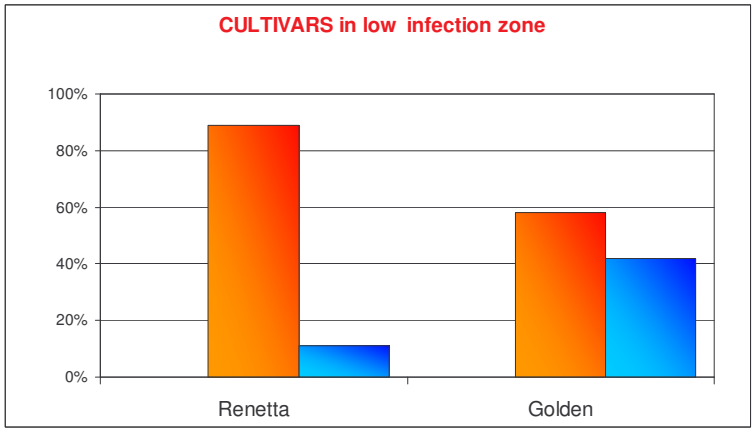


Fig. 36: distribution of the strains in the major rootstocks in 2002. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

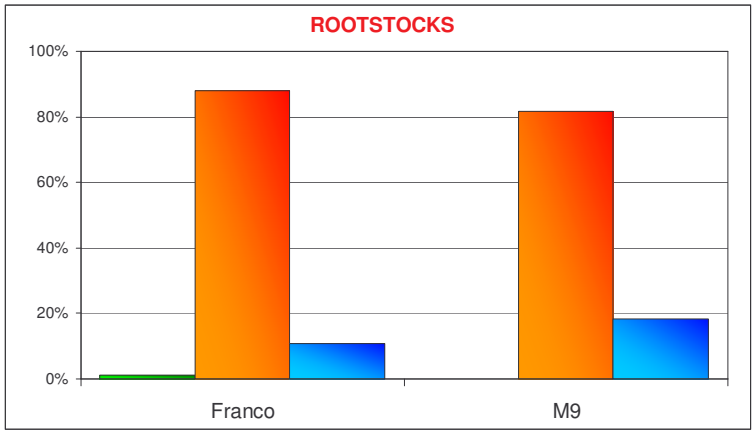
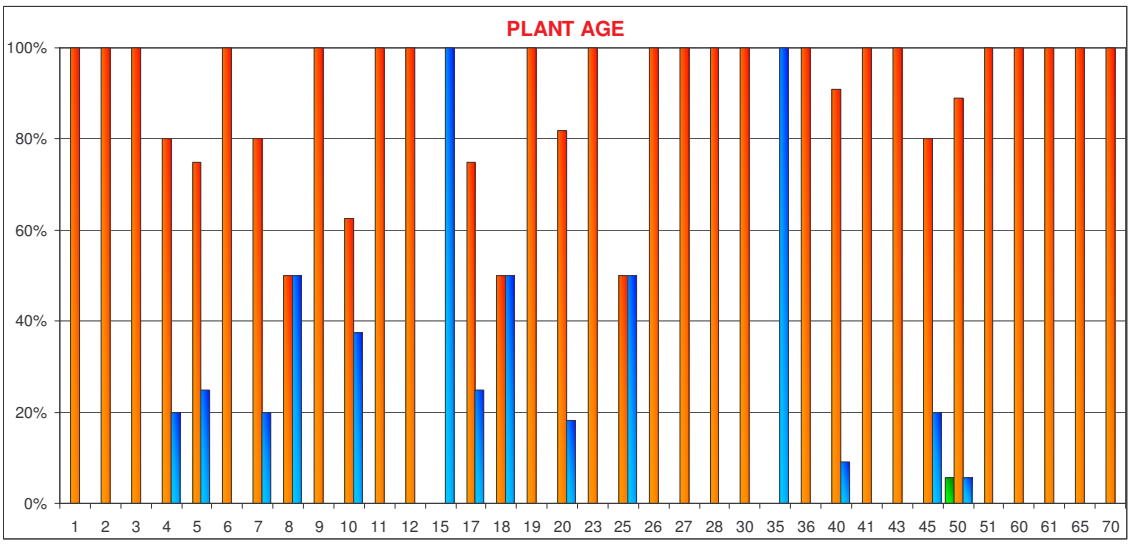


Fig. 37: distribution of the strains according to age of plant samples in 2002. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.



Val di Sole Area

AT2 strain = 22 samples

AT1 strain = 2 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	50	AT2	2
	Franco	60	AT2	2
	Franco	65	AT2	1
	Franco	70	AT2	1
	Franco	17	AT2	1
	Franco	20	AT2	1
	Franco	50	AT2	1
	Franco	50	AT1	1
	M9	4	AT2	1
	M9	6	AT2	1
	M9	7	AT2	1
Golden delicious	Franco	45	AT1	1
	Franco	25	AT2	1
	Franco	28	AT2	1
	Franco	30	AT2	2
	M9	2	AT2	2
	M9	4	AT2	1
	M9	5	AT2	1
	M9	6	AT2	1
Florina	M111	12	AT2	1

Revò - Alta Val di Non Area

AT2 strain = 20 samples

AT1 strain = 0 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	40	AT2	1
	Franco	45	AT2	1
	Franco	50	AT2	3
	Franco	60	AT2	1
	Franco	30	AT2	1
	Franco	40	AT2	2
	M9	6	AT2	1
Golden delicious	Franco	25	AT2	2
	Franco	27	AT2	1
	Franco	30	AT2	1
	Franco	35	AT2	1
	Franco	45	AT2	1
	Franco	20	AT2	1
	Franco	30	AT2	1
	Franco	35	AT2	1
Gala	M9	1	AT2	1

Tuenno - Cles Area

AT2 strain = 21 samples

AT1 strain = 0 samples

AP strain = 1 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	50	AT2	4
	Franco	60	AT2	3
	Franco	50	AP	1
	Franco	20	AT2	1
	Franco	40	AT2	1
	Franco	45	AT2	1
	M9	7	AT2	2
Golden delicious	Franco	30	AT2	2
	Franco	20	AT2	1
	Franco	30	AT2	2
	Franco	40	AT2	1
	M9	3	AT2	1
	M9	5	AT2	1
	M9	10	AT2	1

Taio - Segno Area

AT2 strain = 14 samples

AT1 strain = 0 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	45	AT2	2
	Franco	50	AT2	2
	Franco	40	AT2	1
	Franco	50	AT2	1
	M9	12	AT2	2
	M26	9	AT2	1
Golden delicious	Franco	36	AT2	1
	Franco	45	AT2	1
	Franco	50	AT2	1
	M9	6	AT2	1
	M9	10	AT2	1

Denno - Cunevo - Campodenno Area

AT2 strain = 15 samples

AT1 strain = 2 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	61	AT2	1
	Franco	41	AT2	1
	Franco	51	AT2	2
	M9	8	AT2	1
	M9	11	AT2	1
	M9	12	AT2	1
Golden delicious	Franco	45	AT2	2
	Franco	45	AT1	1
	Franco	?	AT1	1
	Franco	36	AT2	1
	Franco	43	AT2	1
	Franco	?	AT2	1
	M9	4	AT2	1
	M9	10	AT2	1
	M9	17	AT2	1

Val d'Adige Nord Area

AT2 strain = 5 samples

AT1 strain = 1 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	M26	10	AT2	2
Golden delicious	Franco	36	AT2	1
	Franco	25	AT1	1
	M26	30	AT2	1
	Franco	40	AT2	1

Val d'Adige Sud Area

AT2 strain = 3 samples

AT1 strain = 4 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	35	AT1	1
Golden delicious	M9	5	AT1	1
	M26	13	AT2	1
	Franco	26	AT2	1
	Franco	40	AT1	1
	M9	20	AT2	1
Braeburn	M26	10	AT1	1

Valsugana Area

AT2 strain = 10 samples

AT1 strain = 7 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	40	AT2	1
	Franco	50	AT2	2
	Franco	30	AT2	1
	Franco	40	AT2	1
Golden delicious	M106	18	AT1	1
	Franco	40	AT2	1
	Franco	17	AT1	1
	Franco	25	AT1	1
	M9	7	AT2	1
	M9	7	AT1	1
	M11	19	AT2	1
	M26	15	AT1	1
	M106	19	AT2	1
	M106	10	AT1	1
	M9	20	AT2	1
Florina	M106	10	AT1	1

Val del Sarca Area

AT2 strain = 7 samples

AT1 strain = 2 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	M9	5	AT2	1
Golden delicious	M7	23	AT2	1
	M26	20	AT2	1
	M111	30	AT2	1
	M7	20	AT2	1
Jonagold	M9	18	AT2	1
	M9	20	AT1	1
Florina	M26	12	AT2	1
Red delicious	M9	15	AT1	1

Bleggio Area

AT2 strain = 4 samples

AT1 strain = 4 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	M9	20	AT1	1
	M11	25	AT1	1
	M106	30	AT2	1
	M9	4	AT2	1
	M9	4	AT1	1
	M9	8	AT1	1
	M26	17	AT2	1
Jonagold	M106	20	AT2	1

3A.2.1.2 - Second collection

This year 179 and 93 AP positive samples were split up in the 10 areas of Trentino and the 5 micro-areas, respectively. The position of the micro-areas has been established from the results of the first collection.

Therefore the Focus Sole - Cavizzana and Focus Non - Sporminore micro-area were situated in Cavizzana (in Val di Sole area) and Sporminore (in Denno - Cunevo - Campodenno area) respectively, where in the previous year four samples were infected by AT1 strain.

So the Focus Non - Tuenno micro-area was situated in Tuenno (in Tuenno - Cles area) where one sample was infected by AP strain.

The collection of samples in these micro-areas was more intensive and was conducted in a limited area around the plants that were infected by the AT1 or AP strain during the previous year.

Two sites of reclaimed land were chosen in Rumo (Bonifica - Rumo micro-area in Revò - Alta Val di Non area) and Vervò (Bonifica - Vervò micro-area in Taio - Segno area) to verify the situation of the young plants presents there.

3A.2.1.2.1 - Areas

A high presence of AT2 strain was confirmed in this collection (**Fig. 38**). The situation in the valleys of North-West Trentino is almost identical to the previous year. The only strain present in Revò - Alta Val di Non and Val di Sole area was the AT2 strain. Instead, in the zone of Cavizzana in Val di Sole (Focus Non - Cavizzana micro-area) both AT1 and AT2 strains were found. In each one of the other three areas of Val di Non there was only one sample infected with AT1 strain. This confirms the distribution in the Denno - Cunevo - Campodenno area while in the Tuenno - Cles and Taio - Segno area the AT1 strain appears for the first time. AP strain disappeared from Tuenno - Cles area.

The situation in the other areas is slightly different as compared to the previous collection. The distribution in Val d'Adige Nord is identical to the previous sample collection. Even though the presence of AT1 strain in the Val d'Adige Sud, Valsugana, Val del Sarca and Bleggio areas is reduced, it remains higher in respect to that of the North-West Trentino areas. There are not many differences between 2002 and 2004 as regards to the strain distribution in the cultivars (**Fig. 39**) and rootstocks (**Fig. 40**).

The age of the plants was indicated by two intervals of time: from zero to five years and more than five years. Also in this case there is not much of a difference between the two intervals (**Fig. 41**).

3A.2.1.2.2 - Micro-areas

In Focus Sole - Cavizzana micro-area the distribution of the strain is similar to the previous collection (**Fig. 42**). In Focus Non - Sporminore AT1 strain reappears but the AP strain is also present in one sample (**Fig. 42**).

The situation is different in Focus Non - Tuenno micro-area: there's no AP strain any more but two samples are infected with AT1 strain (like the situation of the second collection in Tuenno - Cles area) (**Fig. 42**).

The situation of the Bonifica - Vervò micro-area confirms that of the Taio - Segno area (**Fig. 42**). An AP strain infected sample is present in Bonifica - Rumo. This is a particularity because so far only the AT2 strain was present in Revò - Alta Val di Non area (**Fig. 42**).

Fig: 38: distribution of the strains in the areas in 2004. % AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

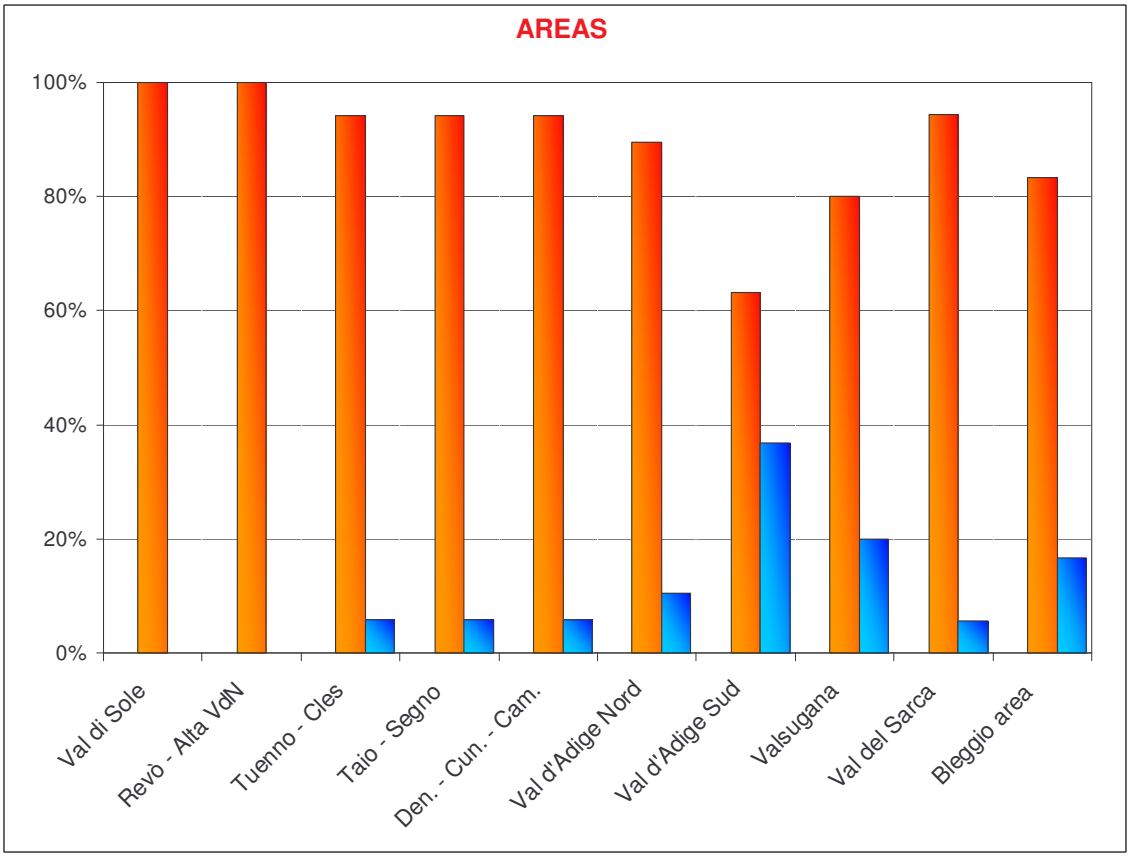


Fig: 39: comparison of the strain distribution in the major cultivars in 2002 and in 2004. % AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

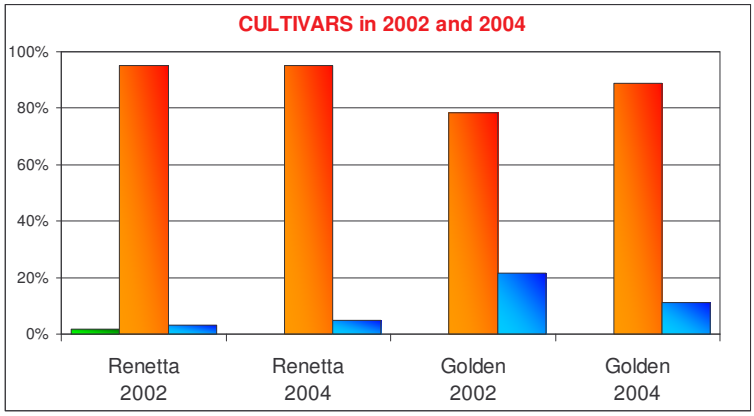


Fig. 40: comparison of the strain distribution in the major rootstocks in 2002 and in 2004. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

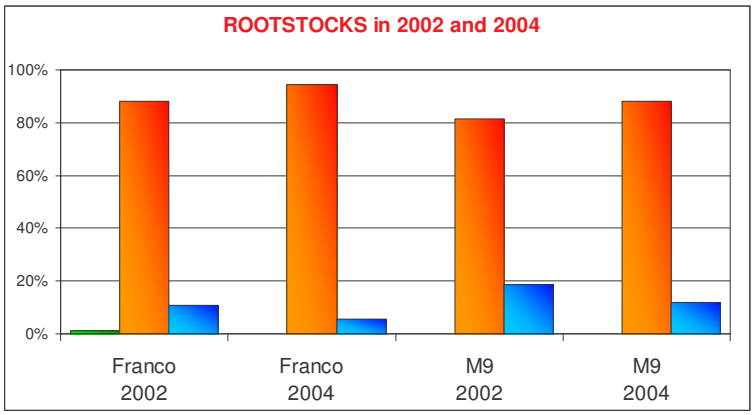


Fig. 41: distribution of the strains according to age interval of plant samples in 2004. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.

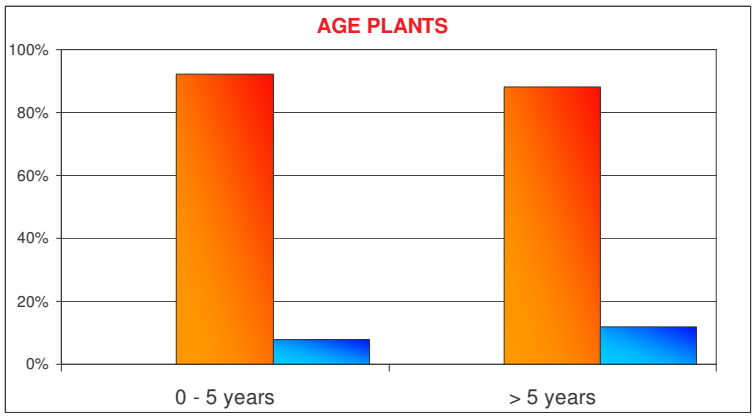
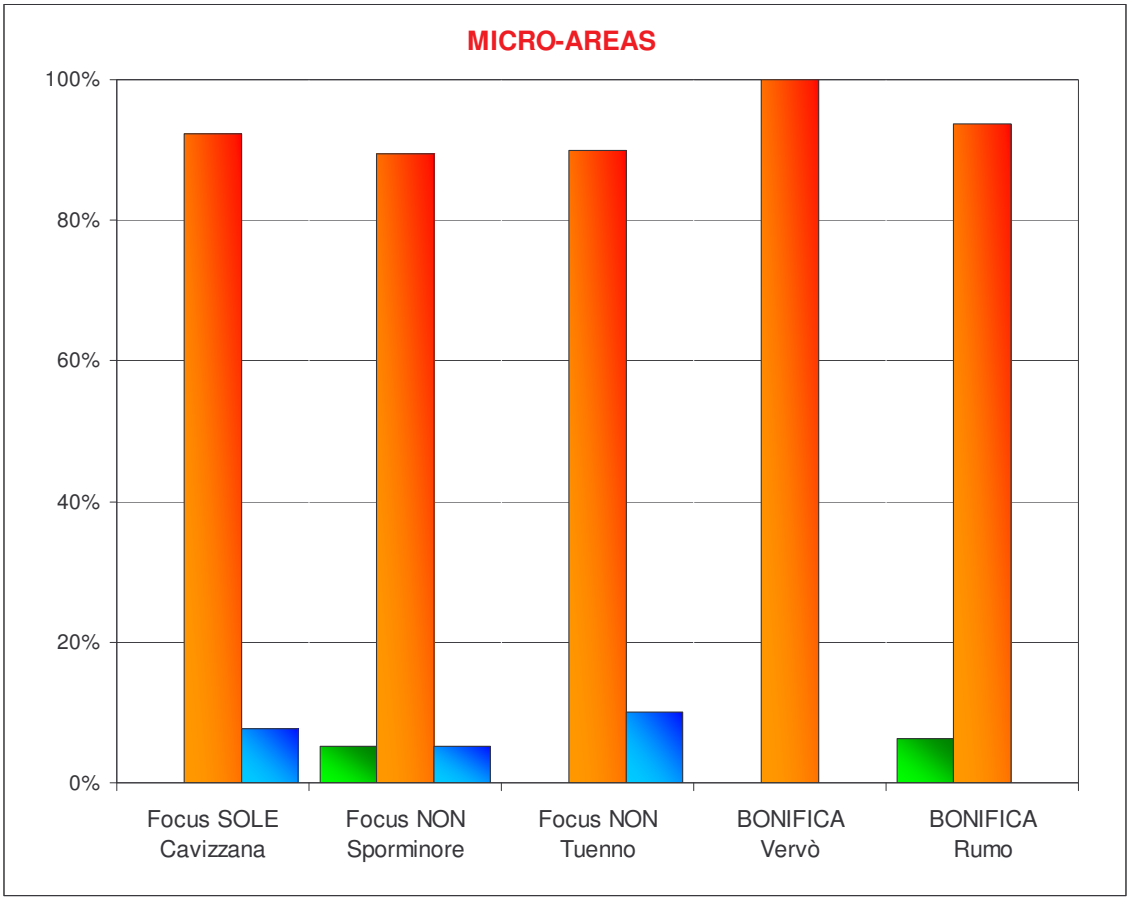


Fig. 42: distribution of the strains in the micro-areas in 2004. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.



Val di Sole Area

AT2 strain = 15 samples

AT1 strain = 0 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	2
	M9	0 - 5	AT2	3
	M9	> 5	AT2	1
Golden delicious	Franco	> 5	AT2	1
	M9	0 - 5	AT2	4
	M9	> 5	AT2	1
	M26	> 5	AT2	1
	M106	> 5	AT2	1
Gala	M9	0 - 5	AT2	1

Revò - Alta Val di Non Area

AT2 strain = 17 samples

AT1 strain = 0 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	4
	M9	0 - 5	AT2	5
Golden delicious	Franco	> 5	AT2	2
	M9	0 - 5	AT2	2
	M26	0 - 5	AT2	1
	M26	> 5	AT2	2
	M7	> 5	AT2	1

Tuenno - Cles Area

AT2 strain = 16 samples

AT1 strain = 1 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	4
	M9	0 - 5	AT2	4
	M9	> 5	AT2	1
Golden delicious	Franco	> 5	AT2	3
	M9	0 - 5	AT2	3
	M9	0 - 5	AT1	1
	M26	> 5	AT2	1

Taio - Segno Area

AT2 strain = 16 samples

AT1 strain = 1 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	4
	Franco	> 5	AT1	1
Golden delicious	Franco	> 5	AT2	4
	M9	0 - 5	AT2	1
	M9	> 5	AT2	1
	M26	> 5	AT2	4
	M106	0 - 5	AT2	1
	M106	> 5	AT2	1

Denno - Cunevo - Campodenno Area

AT2 strain = 16 samples

AT1 strain = 1 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	4
	M9	0 - 5	AT2	1
	M9	> 5	AT2	2
	M26	> 5	AT2	2
Golden delicious	Franco	> 5	AT2	1
	M9	0 - 5	AT2	3
	M9	0 - 5	AT1	1
	M111	0 - 5	AT2	1
	M111	> 5	AT2	1
	M26	> 5	AT2	1

Val d'Adige Nord Area

AT2 strain = 17 samples

AT1 strain = 2 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	M26	> 5	AT2	1
Golden delicious	M9	> 5	AT2	4
	M9	> 5	AT1	1
	M26	> 5	AT2	2
Red delicious	M9	> 5	AT2	3
Gala	M9	0 - 5	AT2	1
	M9	> 5	AT2	1
Granny	M9	> 5	AT2	2
Stayman	M26	> 5	AT1	1
Braeburn	M9	> 5	AT2	1
Idared	M26	> 5	AT2	1
Morgenduft	M111	> 5	AT2	1

Val d'Adige Sud Area

AT2 strain = 12 samples

AT1 strain = 7 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	1
Golden delicious	Franco	> 5	AT2	1
	M9	> 5	AT2	4
	M9	> 5	AT1	4
	M26	> 5	AT2	1
Red delicious	M26	> 5	AT2	1
Granny	M9	> 5	AT2	1
Stayman	M7	> 5	AT2	1
Braeburn	M9	> 5	AT1	1
Morgenduft	M106	> 5	AT2	1
Fuji	M9	0 - 5	AT2	1
Pink lady	M9	0 - 5	AT1	1
Red chief	M26	> 5	AT1	1

Valsugana Area

AT2 strain = 12 samples

AT1 strain = 3 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT1	1
Golden delicious	Franco	> 5	AT2	2
	M9	> 5	AT2	2
	M26	> 5	AT2	3
	M26	> 5	AT1	1
Red delicious	M9	> 5	AT2	1
	M106	> 5	AT1	1
Gala	M9	0 - 5	AT2	1
	M9	> 5	AT2	1
Red chief	M26	0 - 5	AT2	1
Morgenduft	M106	> 5	AT2	1

Val del Sarca Area

AT2 strain = 17 samples

AT1 strain = 1 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	M9	0 - 5	AT2	1
	M9	> 5	AT2	6
	M26	> 5	AT1	1
	M11	> 5	AT2	1
Red delicious	M9	> 5	AT2	5
Gala	M9	0 - 5	AT2	1
	M9	> 5	AT2	2
Morgenduft	M26	> 5	AT2	1

Bleggio Area

AT2 strain = 10 samples

AT1 strain = 2 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	M9	> 5	AT2	4
	M26	> 5	AT2	4
	M106	> 5	AT2	2
	M106	> 5	AT1	1
Red delicious	M9	> 5	AT1	1

FOCUS Sole - Cavizzana Micro-area

AT2 strain = 12 samples

AT1 strain = 1 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	Franco	> 5	AT2	5
	Franco	> 5	AT1	1
	M9	> 5	AT2	6
?	M9	> 5	AT2	1

FOCUS Non - Sporminore Micro-area

AT2 strain = 17 samples

AT1 strain = 1 samples

AP strain = 1 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	Franco	> 5	AT2	13
	Franco	> 5	AT1	1
	Franco	> 5	AP	1
	M26	> 5	AT2	3
	M106	> 5	AT2	1

FOCUS Non - Tuenno Micro-area

AT2 strain = 18 samples

AT1 strain = 2 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	> 5	AT2	5
Golden delicious	Franco	> 5	AT2	1
	Franco	> 5	AT1	1
	M9	> 5	AT2	2
	M11	> 5	AT2	5
	M11	> 5	AT1	1
	M106	> 5	AT2	4
	M111	> 5	AT2	1

BONIFICA - Rumo Micro-area

AT2 strain = 15 samples

AT1 strain = 0 samples

AP strain = 1 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	M9	0 - 5	AT2	11
	M9	0 - 5	AP	1
Lasa	M9	0 - 5	AT2	3
Red delicious	M9	0 - 5	AT2	1

BONIFICA - Vervò Micro-area

AT2 strain = 17 samples

AT1 strain = 0 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	M9	0 - 5	AT2	2
Golden delicious	M9	0 - 5	AT2	8
Fuji	M9	0 - 5	AT2	4
Gala	M9	0 - 5	AT2	1
Red delicious spur	M9	0 - 5	AT2	1
Sansa	M9	0 - 5	AT2	1

3A.2.1.3 - Third collection

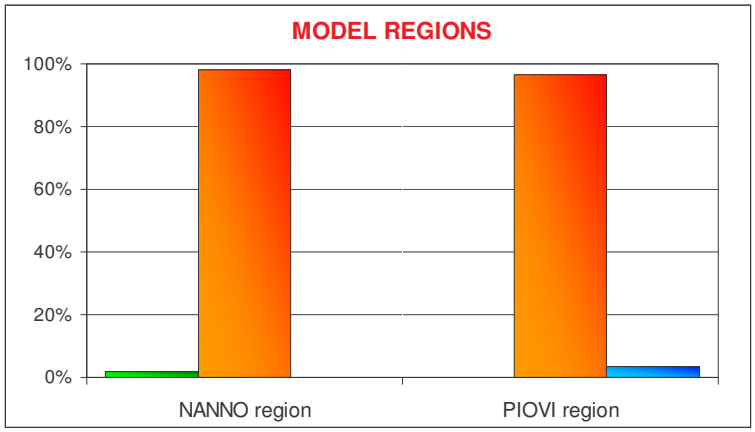
In the model region of Nanno and Piovi 356 AP positive samples were present.

The Nanno region is located in the Tuenno - Cles area. Here, the former collection of AP and AT1 strains appeared in some samples. The analysis confirms the massive presence of AT2 strain and the AP strain (only 4 samples) (**Fig. 43**). In three years of collection AP strain appeared only in three areas of Val di Non: in Revò - Alta Val di Non (in the reclaimed land of Bonifica - Rumo micro-area), in Tuenno - Cles area (in the first and third collection) and in Denno - Cunevo - Campodenno area (in the Focus Non - Sporminore micro-area). No AT1 strain was present, although the previous year it was present in the Tuenno - Cles area.

The Piovi region is located in Val d'Adige Nord, where in the previous years some samples infected with AT1 strain were always present. The analysis confirms the following: a massive presence of AT2 strain and only 5 samples of AT1 strain (**Fig. 43**). The AP strain is completely absent. The situation in the areas of Central and South Trentino are similar but the percentage of the distribution of the two strains is slightly variable.

Seeing the characteristic distribution of strains in the model regions the data is not significant as regards to the cultivars, the rootstocks and the age of the plants.

Fig. 43: distribution of the strains in the model regions in 2005. AP strain = green; % AT2 strain = orange; % AT1 strain = blue.



Model Region Nanno (Tuenno - Cles area)

AT2 strain = 196 samples

AT1 strain = 0 samples

AP strain = 4 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Renetta canada	Franco	35	AT2	3
	Franco	55	AT2	23
	Franco	57	AT2	20
	Franco	59	AT2	1
	Franco	60	AT2	1
	Franco	61	AT2	1
	Franco	62	AT2	1
	Franco	63	AT2	68
	Franco	70	AT2	17
	Franco	57	AP	1
	Franco	63	AP	1
	M11	25	AT2	2
	M106	15	AT2	1
Golden delicious	Franco	53	AT2	33
	Franco	53	AP	1
	M9	9	AT2	13
	M9	9	AP	1
	M11	22	AT2	3
	M26	16	AT2	3
Red delicious	Franco	25	AT2	1
	Franco	53	AT2	2
	Franco	63	AT2	2
	M11	25	AT2	1

Model Region Piovi (Val d'Adige Nord area)

AT2 strain = 135 samples

AT1 strain = 5 samples

AP strain = 0 samples

CULTIVAR	ROOTSTOCK	PLANT AGE	STRAIN	N°
Golden delicious	M9	7	AT2	12
	M9	10	AT2	11
	M9	16	AT2	22
	M9	18	AT2	6
	M9	7	AT1	1
	M9	10	AT1	2
	M9	16	AT1	1
	M26	12	AT2	1
Gala	M9	3	AT2	1
	M9	10	AT2	47
Red delicious	M9	6	AT2	5
	M9	10	AT2	6
	M9	14	AT2	1
	M9	16	AT2	4
Braeburn	M9	9	AT2	11
Granny	M9	0	AT2	3
	M9	0	AT1	1
Fuji	M9	5	AT2	2
	M9	9	AT2	1
Morgenduft	M9	16	AT2	2

3A.2.2 - Psyllid samples

The only variables considered during the strain analysis performed on 262 AP positive samples collected during three years are the following:

- area of origin of the sample,
- species,
- developmental stages.

3A.2.2.1 - First collection

In the first year 162 AP positive samples were split up in 4 areas of North-West Trentino (high infection zone) and in 4 areas of Central and South Trentino (low infection zone). Insects have not been collected in the Taio - Segno and in Bleggio area. The analysis roughly confirms the distribution of strains in the plants in year 2002 (**Fig. 44**).

In Val di Sole area all the insects are infected by the AT2 strain but in Cavizzana none of these insects were collected. Therefore the situation of the insects and the plants is the same in the Val di Sole area.

Also in Tuenno - Cles area the strain distribution in the insects is the same as that of the plants. AT2 strain is the most widespread and one sample is infected by AP strain (near Cles, not Tuenno as in the case of the plants).

Only the AT2 strain was present in Denno - Cunevo - Campodenno area while the plants were also infected with AT1 in the first collection.

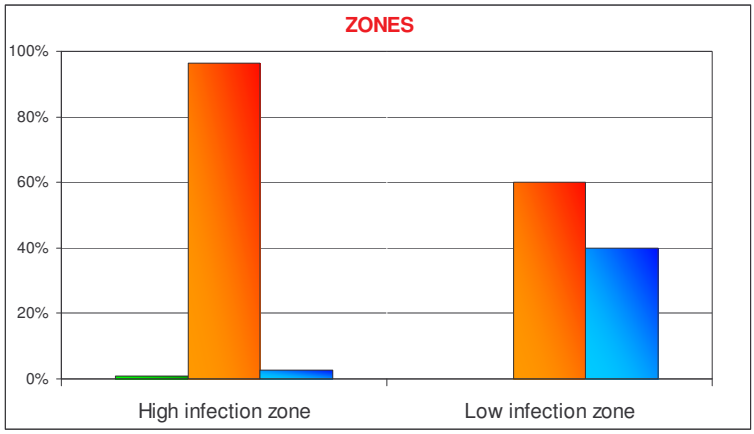
Besides the AT2 strain, the novelty is the presence of AT1 strain in the three samples of *C. picta* collected in the Revò - Alta Val di Non area.

The insect samples collected in other areas of Trentino (low infection zone) are very few. But the analysis shows that the strain distribution in the insect is the same as that of the plant in these areas in first collection. AP strain is completely absent.

Since nearly all the samples of *C. picta* and *C. melanoneura* come from the high infection zone and low infection zone, respectively, the distribution of strains in the species is the same as that found in the zone.

The data of the stage of development does not give any significant information.

Fig. 44: distribution of the strains in the psyllids of the high infection and low infection zones in 2002. % AP strain = green; % AT2 strain = orange; % AT1 strain = blue.



AT2 strain = 116 samples

AT1 strain = 9 samples

AP strain = 1 samples

AREA	SPECIES	STAGE	STRAIN	N°
Val di Sole	*C. melanoneura*	Overwintering	AT2	1
	C. picta	Overwintering	AT2	5
	C. mali	New Adult	AT2	1
Revò - Alta Val di Non	*C. picta*	Overwintering	AT2	37
	C. picta	Overwintering	AT1	3
Tuenno - Cles	*C. picta*	Overwintering	AT2	21
	C. picta	Overwintering	AP	1
Denno - Cunevo - Campodenno	*C. melanoneura*	Overwintering	AT2	1
	C. picta	Overwintering	AT2	41
Val d'Adige Nord	*C. melanoneura*	Overwintering	AT2	1
	C. melanoneura	Overwintering	AT1	2
	C. melanoneura	Young	AT2	1
	C. picta	Overwintering	AT2	3
Val d'Adige Sud	*C. melanoneura*	Overwintering	AT2	1
	C. melanoneura	Overwintering	AT1	2
	C. picta	Overwintering	AT2	1
Valsugana	*C. melanoneura*	Overwintering	AT2	2
	C. melanoneura	Overwintering	AT1	1
Val del Sarca	*C. melanoneura*	Overwintering	AT1	1

3A.2.2.2 - Second collection

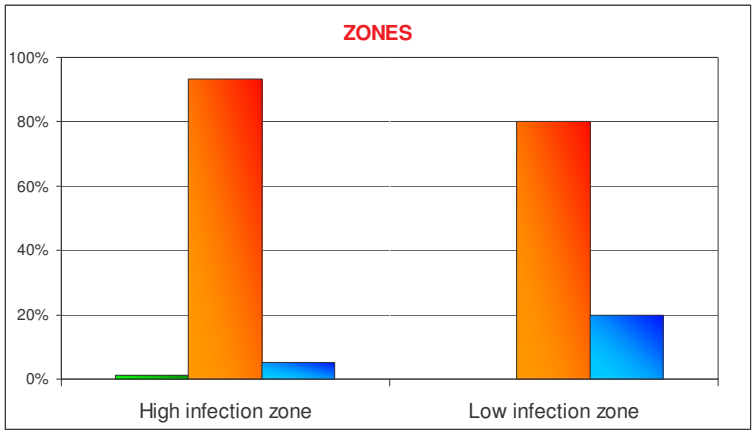
The infected samples collected in this year were very few (only 100). Only in two areas there were a good number of samples analysed. In both, the AT2 strain is present the most. However, also in this case the analysis roughly confirms the distribution of strains in the plants in the same year (**Fig. 45**).

In the Revò - Alta Val di Non area the AT1 strain is present in the same zone were the collection was made in the previous year. But the AP strain is also present and exactly in the same zone where there is the AT1 strain.

The diffusion of the strain in the insect in the Tuenno - Cles area is the same as that of the plant in the year 2004. The AT1 strain took the place of the AP strain.

The data of the strain distribution in the species and in the stage of development are not informative.

Fig. 45: distribution of the strains in the psyllids of the high infection and low infection zones in 2004. % AP strain = green; % AT2 strain = orange; % AT1 strain = blue.



AT2 strain = 74 samples

AT1 strain = 5 samples

AP strain = 1 samples

AREA	SPECIES	STAGE	STRAIN	N°
Revò - Alta Val di Non	*C. picta*	Overwintering	AT2	39
	C. picta	Overwintering	AT1	1
	C. picta	Overwintering	AP	1
	C. picta	Young	AT2	3
Tuenno - Cles	*C. picta*	Overwintering	AT2	26
	C. picta	Overwintering	AT1	2
	C. picta	Young	AT2	1
	C. melanoneura	New Adult	AT1	1
Denno - Cunevo - Campodenno	*C. picta*	Overwintering	AT2	1
Val d'Adige Nord	*C. picta*	Overwintering	AT2	4
	C. melanoneura	Overwintering	AT1	1

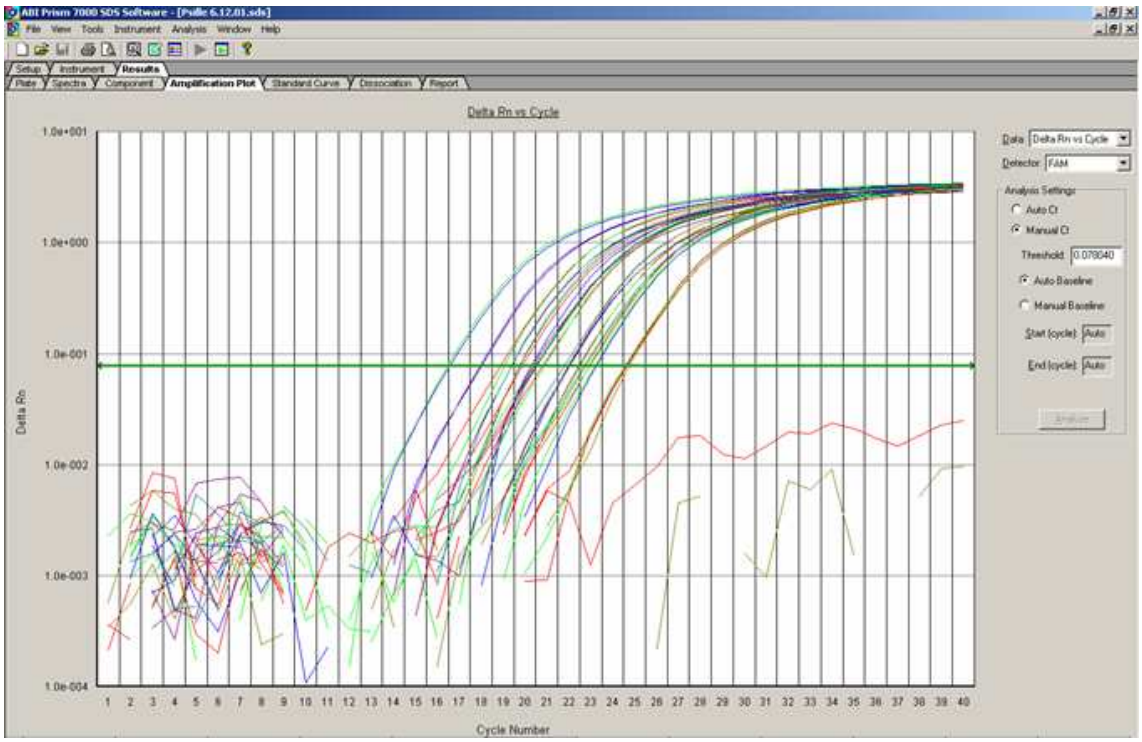
3A.2.2.3 - Third collection

Unfortunately, the strain analysis on only four infected samples was unsuccessful.

3A.3 - QUANTITATIVE ANALYSIS

After the strain analysis, a given group of samples underwent quantitative PCR analysis (**Fig. 46**). This analysis allows quantify the phytoplasma in the sample. The used technique is always the same with all the different kinds of samples (plant or insect) but the measurement of the quantity of pathogen changes according to type of sample.
The analysis of variance was conducted on the quantitative data. In addition, the regression analysis was conducted to compare the quantitative data with the age of the plant.

Fig. 46: example of quantitative PCR analysis.



3A.3.1 - Plant samples

The plant samples subjected to quantitative analysis must be as much as possible homogeneous so that the resultant data may be compared between them. The data are expressed in "n° of phytoplasma copies on one pg of the DNA of the plant tissue". Therefore it's important that the leaves of the various samples are collected from the same kind of branch, in the same period and that the DNA extraction is the same for all the samples.

The analysis was carried out on 76 and 84 samples of Nanno and Piovi model region, respectively (**Tab. 24-25**). The samples are constituted by the main nervation of leaf originated from the apical branch of each plant. They have been collected during a period of 9 days.

The q-PCR was conducted only on these samples because they produced more information and the data collected was compared with others data similar to these (the protocols of collection, DNA extraction and the analyses have been very homogeneous for all the samples).

Tab. 24: plant samples of the Nanno region analysed by q-PCR.

CULTIVAR	ROOTSTOCK	STRAIN	PLANT AGE	n° Phyto. / pg DNA
Renetta canada	Franco	AT2	57	348,7
		AT2	57	91,3
		AT2	57	33,5
		AT2	57	415,1
		AT2	63	29,4
		AT2	63	26,5
		AT2	59	264,1
		AT2	63	277,5
		AT2	63	148,1
		AT2	63	349,4
		AT2	63	304,2
		AT2	63	150,6
		AT2	57	232,9
		AT2	63	352,0
		AT2	63	81,2
		AT2	63	76,6
		AT2	63	272,0
		AT2	70	105,8
		AT2	55	162,8
		AT2	55	70,5
		AT2	55	202,4
		AT2	55	158,1
		AT2	55	79,2
Renetta canada	Franco	AP	57	55,3
		AP	63	130,1
Renetta canada	M11	AT2	25	60,2
		AT2	25	245,9
Renetta canada	M106	AT2	15	151,2
Golden delicious	Franco	AT2	53	187,4
		AT2	53	130,7
		AT2	53	232,5
		AT2	53	482,2
		AT2	53	285,1
		AT2	53	216,4
		AT2	53	68,6
		AT2	53	152,6
		AT2	53	138,7
		AT2	53	258,0
		AT2	53	195,5

Golden delicious	Franco	AT2	53	75,8
		AT2	53	112,8
		AT2	53	227,7
		AT2	53	192,0
		AT2	53	189,8
		AT2	53	31,6
		AT2	53	29,5
		AT2	53	109,5
		AT2	53	184,7
		AT2	53	588,0
Golden delicious	Franco	AP	53	75,7
Golden delicious	M9	AT2	9	154,2
		AT2	9	606,8
		AT2	9	1.079,9
		AT2	9	785,9
		AT2	9	662,0
		AT2	9	348,5
		AT2	9	153,9
		AT2	9	744,6
		AT2	9	359,2
		AT2	9	1.840,4
		AT2	9	264,9
		AT2	9	22,1
		AT2	9	421,4
Golden delicious	M9	AP	9	141,4
Golden delicious	M11	AT2	22	149,2
		AT2	22	23,6
		AT2	22	234,8
Golden delicious	M26	AT2	16	217,6
		AT2	16	401,7
		AT2	16	145,7
Red delicious	Franco	AT2	63	188,7
		AT2	63	190,1
		AT2	53	121,2
		AT2	53	162,6
		AT2	25	141,1
Red delicious	M11	AT2	25	56,1

Tab. 25: plant samples of the Piovi region analysed by q-PCR.

CULTIVAR	ROOTSTOCK	STRAIN	PLANT AGE	n° Phyto. / pg DNA
Golden delicious	M9	AT2	7	104,3
		AT2	7	133,2
		AT2	7	94,7
		AT2	7	136,7
		AT2	7	67,7
		AT2	16	92,0
		AT2	16	157,3
		AT2	16	831,6
		AT2	16	33,7
		AT2	16	12,6
		AT2	16	58,4
		AT2	16	33,5
		AT2	16	856,2
		AT2	16	1326,4
		AT2	18	625,1
		AT2	18	227,8
		AT2	18	187,2
		AT2	10	60,4
		AT2	10	305,6
		AT2	10	270,8
		AT2	10	85,7
		AT2	10	726,5
		AT2	10	592,8
Golden delicious	M9	AT1	7	34,5
		AT1	16	1162,0
		AT1	10	644,2
		AT1	10	317,8
Golden delicious	M26	AT2	12	12,7
Gala	M9	AT2	3	30,2
		AT2	10	117,0
		AT2	10	193,1
		AT2	10	56,0
		AT2	10	231,0
		AT2	10	287,9
		AT2	10	63,0
		AT2	10	240,1
		AT2	10	103,6
		AT2	10	5,1
		AT2	10	176,4
		AT2	10	86,1
		AT2	10	283,1
		AT2	10	55,4
		AT2	10	10,0
		AT2	10	62,3
		AT2	10	55,4
		AT2	10	220,3
		AT2	10	44,3
		AT2	10	21,1
Red delicious	M9	AT2	6	436,1
		AT2	6	100,2
		AT2	6	29,2
		AT2	6	130,8
		AT2	6	160,2
		AT2	16	51,1
		AT2	16	542,0

Red delicious	M9	AT2	16	80,9
		AT2	16	656,3
		AT2	14	196,9
		AT2	10	103,5
		AT2	10	25,4
		AT2	10	101,8
		AT2	10	43,3
		AT2	10	324,9
		AT2	10	450,2
Braeburn	M9	AT2	9	88,3
		AT2	9	342,4
		AT2	9	123,2
		AT2	9	25,0
		AT2	9	51,2
		AT2	9	18,6
		AT2	9	48,0
		AT2	9	504,4
		AT2	9	11,7
		AT2	9	56,7
		AT2	9	406,5
Granny	M9	AT2	0	35,6
		AT2	0	84,0
		AT2	0	127,4
Granny	M9	AT1	0	137,9
Fuji	M9	AT2	5	17,4
		AT2	5	0,9
		AT2	9	82,2
Morgenduft	M9	AT2	16	36,0
		AT2	16	246,4

3A.3.1.1 - Strains

According to the analysis of variance the average values of phytoplasma concentration in the Piovi and in the Nanno region are alike. Furthermore in Nanno the concentration of the AT2 strain is more than the AP strain, on an average. There is not any significant difference (**Fig. 47**). In Piovi the concentration of AT1 strain in the plants is significant different (is higher) from that of the AT2 strain (**Fig. 48**)

Fig. 47: average concentration of AP and AT2 strains present in the plants in the Nanno region.

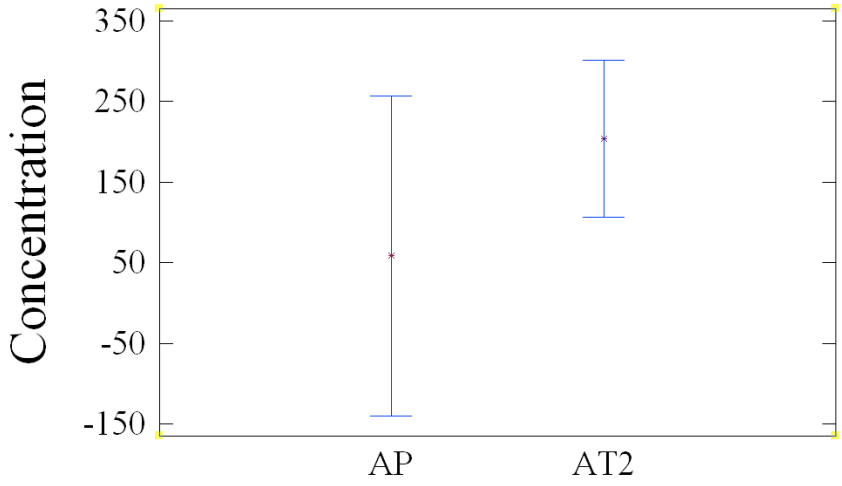
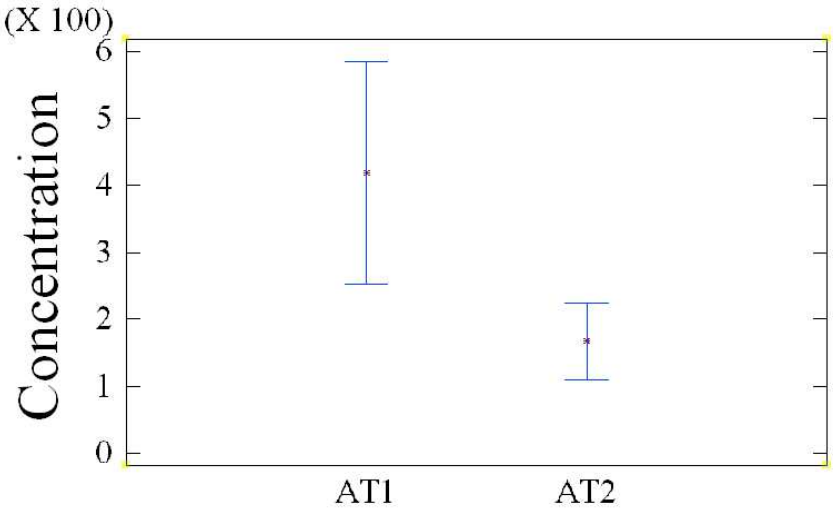


Fig. 48: average concentration of AT1 and AT2 strains present in the plants in the Piovi region.



3A.3.1.2 - Cultivars

Inside each region there is not any significant difference between the cultivars.

The average concentration of phytoplasma in the *Golden delicious* is higher in the Nanno region (**Fig. 49**).

In the Piovi region the highest concentration of phytoplasma is found in *Granny* (**Fig. 50**).

Golden and *Red delicious* plants have a higher concentration of phytoplasma in Piovi as compared to Nanno.

Fig. 49: average phytoplasmal concentration in the cultivars of the Nanno region.

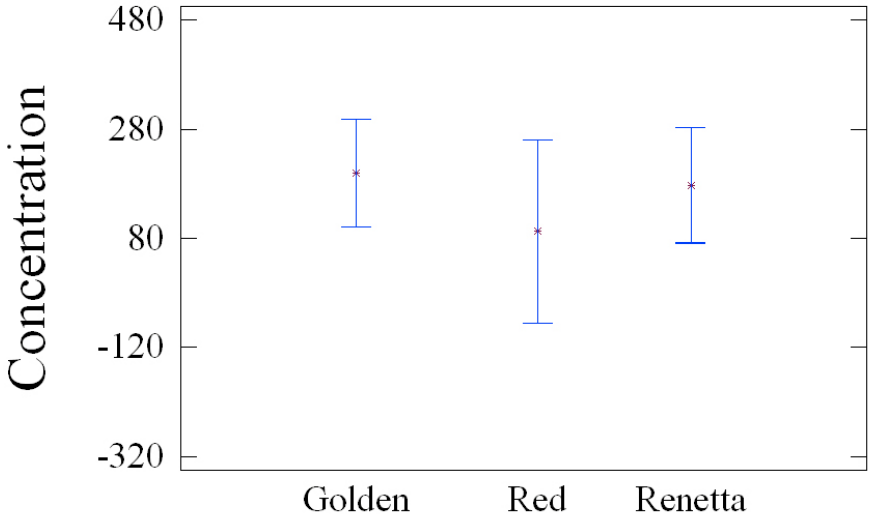
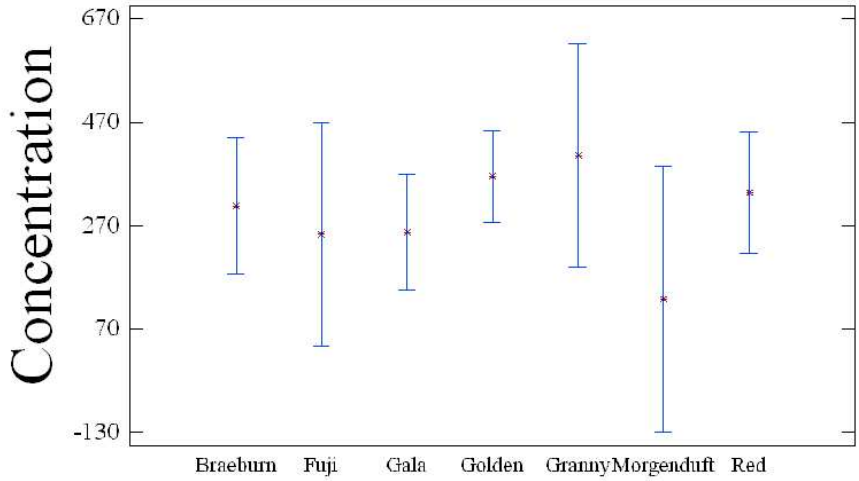


Fig. 50: average phytoplasmal concentration in the cultivars of the Piovi region.

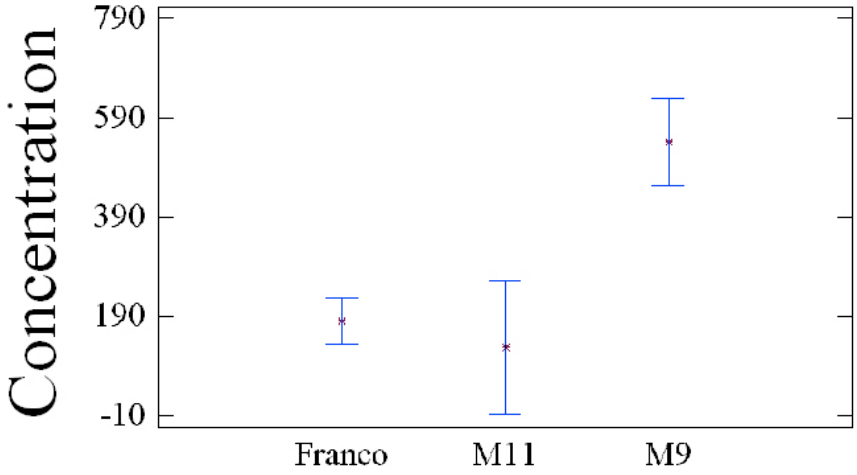


3A.3.1.3 - Rootstocks

In Piovi all the samples analysed have M9 rootstock.

According to the analysis of variance in Nanno the phytoplasmal concentration in the plants with M9 rootstock is significantly different (is higher) from that in the plants with Franco or M11 rootstocks (**Fig. 51**).

Fig. 51: average phytoplasmal concentration in the plants with some rootstocks in the Nanno region.



3A.3.1.4 - Age

In the Nanno and Piovi regions the plants that are 9 and 16 years old, respectively, have a higher average concentration. There are significant differences between the various ages of the plants (**Fig. 52-53**).

According to the regression analysis, in Nanno the concentration decrease with the age, while in Piovi it is the opposite (**Fig. 54-55**).

Fig. 52: average phytoplasmal concentration in the plants of different ages in the Nanno region.

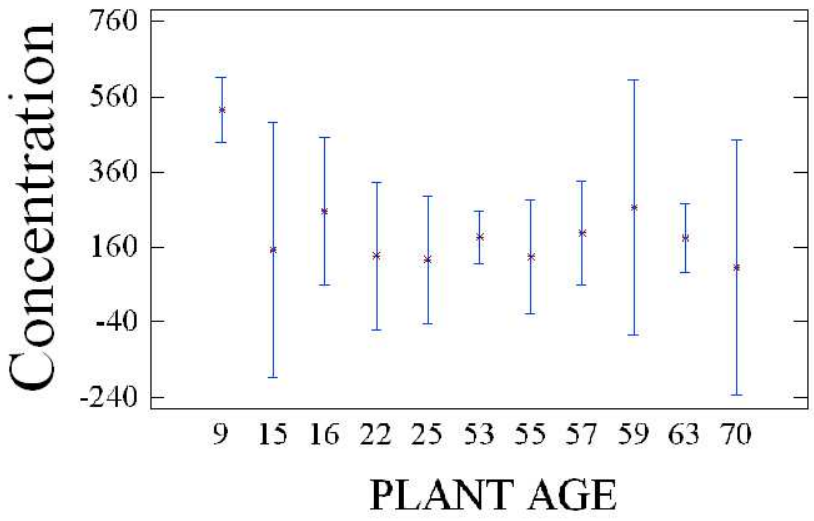


Fig. 53: average phytoplasmal concentration in the plants of different ages in the Piovi region.

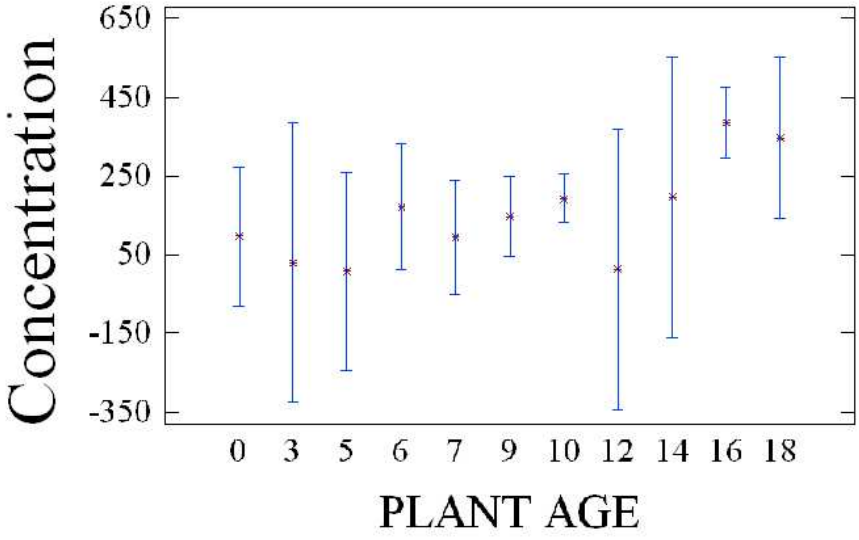


Fig. 54: regression analysis with the variables "plant age" and "concentration of phytoplasma" in the samples of the Nanno region.

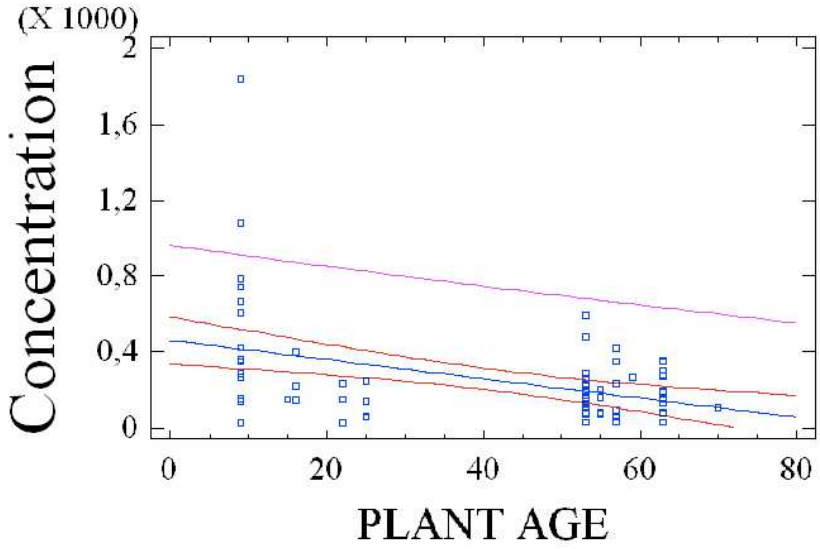
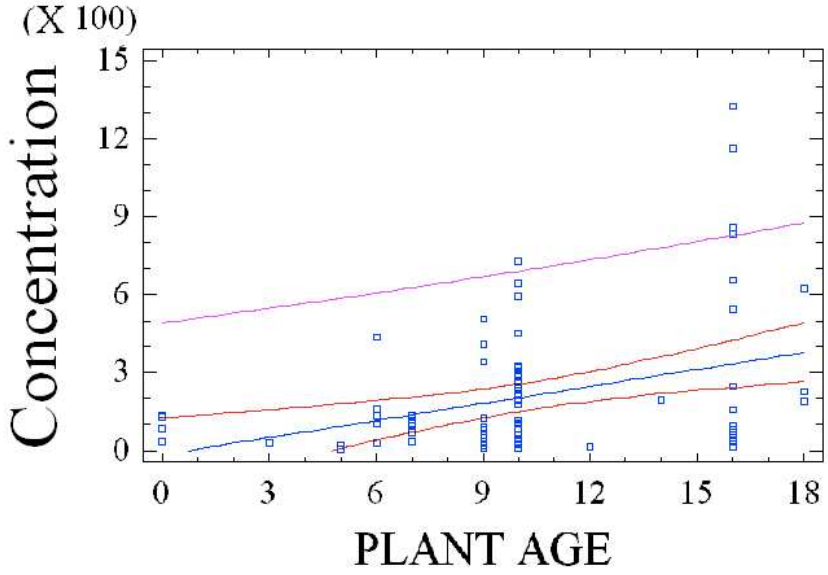


Fig. 55: regression analysis with the variables "plant age" and "concentration of phytoplasma" in the samples of the Piovi region.



3A.3.2 - Psyllid samples

The data of the quantitative analysis on infected insects are expressed in "n° of phytoplasma copies on one insect". Therefore it's important that the analysed sample is constituted by only one insect.

Consequently, the analysis was carried out on only 9 and 32 samples of the 2002 and 2004 collection, respectively (**Tab. 26-27**). Most of the insects come from the Revò - Alta Val di Non area.

Tab. 26: q-PCR analysis of psyllid samples captured in the year 2002.

AREA	SPECIES	STAGE	STRAIN	n° Phyto. / insect
Val di Sole	C. melanoneura	Overwintering	AT2	9.474
Val d'Adige Nord	C. picta	Overwintering	AT2	22.308.618
Val d'Adige Nord	C. melanoneura	Overwintering	AT2	278.750.000
Val d'Adige Nord	C. melanoneura	Overwintering	AT1	46.750.000
Val d'Adige Sud	C. picta	Overwintering	AT2	131.500.000
Val d'Adige Sud	C. melanoneura	Overwintering	AT1	21.625.000
Valsugana	C. melanoneura	Overwintering	AT2	2.303.571
Valsugana	C. melanoneura	Overwintering	AT1	36.000.000
Val del Sarca	C. melanoneura	Overwintering	AT1	13.025.000

Tab. 27: q-PCR analysis of psyllid samples captured in the year 2004.

AREA	SPECIES	STAGE	STRAIN	n° Phyto. / insect
Revò - Alta Val di Non	C. picta	Overwintering	AT2	35.275
		Overwintering	AT2	6.966.490
		Overwintering	AT2	7.550.684
		Overwintering	AT2	10.823.858
		Overwintering	AT2	16.950.000
		Overwintering	AT2	18.990.931
		Overwintering	AT2	26.500.000
		Overwintering	AT2	28.500.000
		Overwintering	AT2	48.500.000
		Overwintering	AT2	77.250.000
		Overwintering	AT2	87.250.000
		Overwintering	AT2	98.000.000
		Overwintering	AT2	107.750.000
		Overwintering	AT2	120.500.000
		Overwintering	AT2	125.500.000
		Overwintering	AT2	182.250.000
		Overwintering	AT2	191.000.000
		Overwintering	AT2	214.500.000
		Overwintering	AT2	242.250.000
		Overwintering	AT2	252.500.000
		Overwintering	AT2	280.000.000
		Overwintering	AT2	280.000.000
		Overwintering	AT2	2.025.000.000
Revò - Alta Val di Non	C. picta	Overwintering	AT1	700.000.000
Revò - Alta Val di Non	C. picta	Nymph	AT2	13.634.242
		Nymph	AT2	53.000.000
		Nymph	AT2	213.500.000
Tuenno - Cles	C. melanoneura	New Adult	AT1	4.325.000
Val d'Adige Nord	C. picta	Overwintering	AT2	50.125.000
		Overwintering	AT2	140.250.000
		Overwintering	AT2	1.115.000.000
Val d'Adige Nord	C. melanoneura	Overwintering	AT1	13.750.000

The data is not much and the analysis does not result significant. But it should be noted that a higher concentration is present in 2004 as regards to 2002 (**Fig. 56**), and in *C. picta* as regards to *C. melanoneura* (**Fig. 57**). The average concentration of AT1 strain is higher (**Fig. 58**).
In 2004 the pathogen concentration of *C. picta* in the Val d'Adige Nord area is higher than in the Revò - Alta Val di Non area (**Fig. 59**).

Fig. 56: average phytoplasmal concentration in the psyllid samples captured in the year 2002 and 2004.

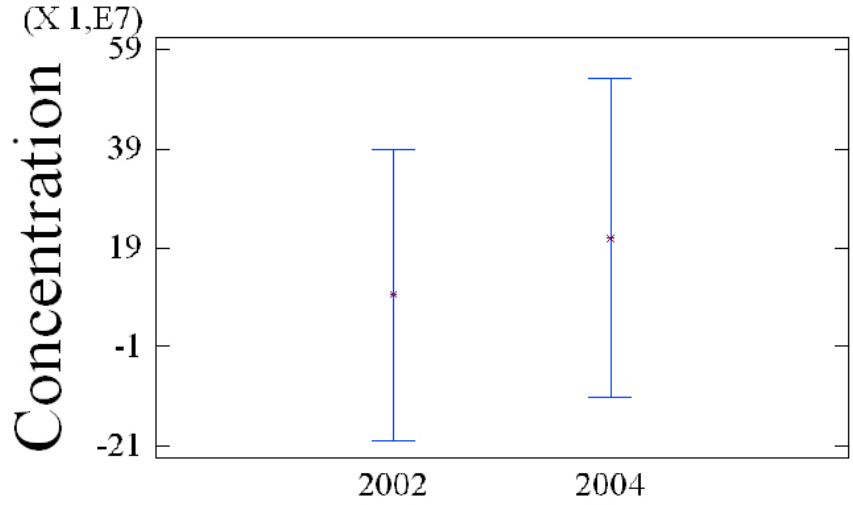


Fig. 57: average phytoplasmal concentration in the different species of psyllids.

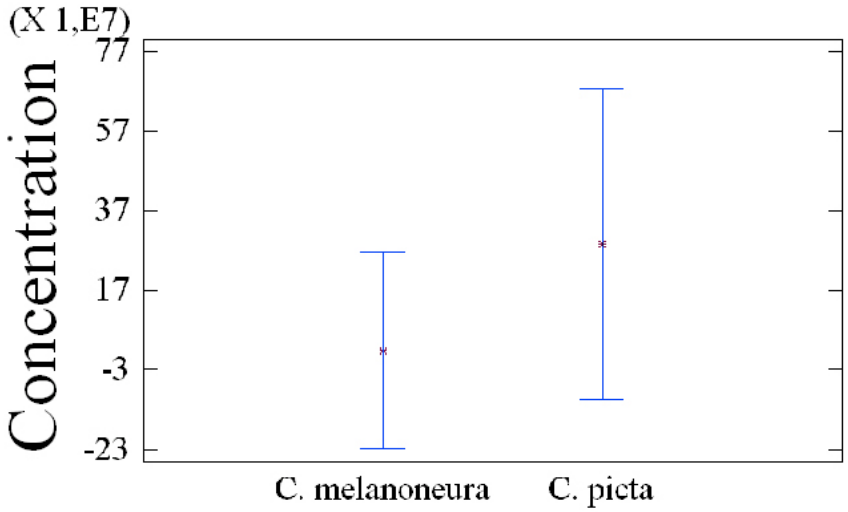


Fig. 58: average concentration of AT1 and AT2 strains present in the psyllids captured in the year 2002 and 2004.

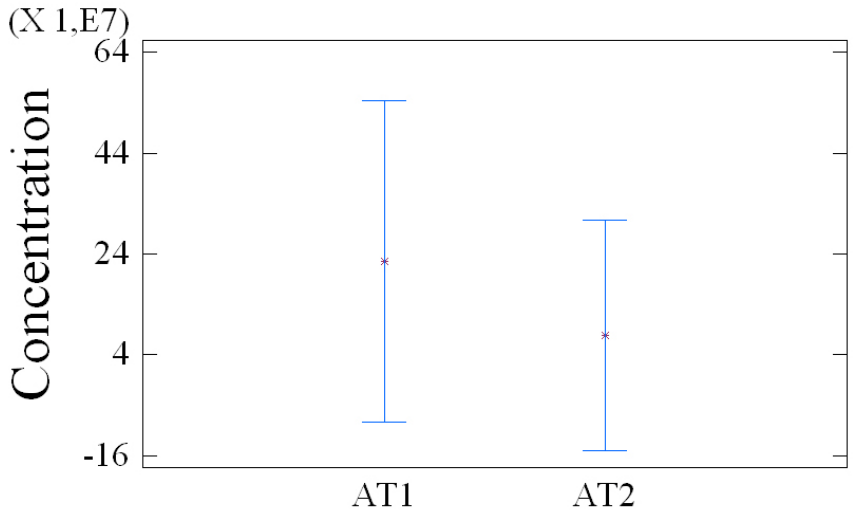
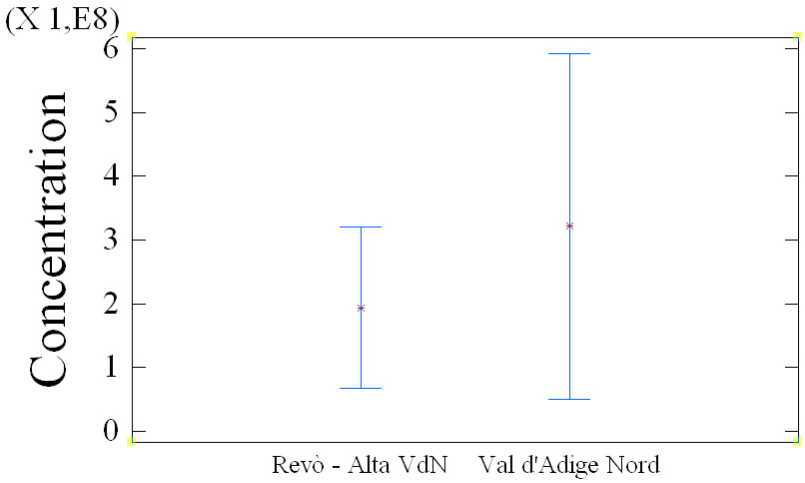


Fig. 59: average phytoplasmal concentration present in *C. picta* captured in the Revò - Alta Val di Non and Val d'Adige Nord areas in 2004.



3B - AP TRANSMISSION CAPACITY OF APHIDS IN TRENTINO

3B.1 - PHYTOPLASMA DETECTION

Aphids, like the psyllids, were picked up and divided according to their species. In the first three years 981 insects were captured while in 2006 only 42 samples were used to establish the quantity of phytoplasma in different parts of the aphids. All the analyses were conducted on a single insect. The analysis of variance was conducted on the quantitative data.

3B.1.1 - 2003 collection

In the first year 109 out of 193 aphid samples were resulted *Ca.* Phytoplasma mali positive. *A. pomi* was present in two areas of Val di Non (Tuenno - Cles and Denno - Cunevo - Campodenno areas) and the Val d'Adige Nord area while *D. plantaginea* was present only in those of Val di Non (**Tab. 28**).

Tab. 28: analysis of the aphids samples captured in 2003.

AREA	SPECIES	N° SAMPLES	N° SAMPLES INFECTED
Tuenno - Cles	*Dysaphis plantaginea*	6	5
	Aphis pomi	15	7
Taio - Segno	*Dysaphis plantaginea*	45	15
Denno - Cunevo - Campodenno	*Dysaphis plantaginea*	21	6
	Aphis pomi	36	31
Val d'Adige Nord	*Aphis pomi*	70	45
TOTAL		193	109

3B.1.2 - 2004 collection

The insects were captured only in two areas of Val di Non and in Val d'Adige Nord area. The presence of four species in Val d'Adige Nord was noted: *D. plantaginea*, *A. pomi*, *D. devecta* and, *R. insertum*. The first two species were the most numerous.

In this collection only 14 out of 133 aphid samples were AP positive (**Tab. 29**).

Tab. 29: analysis of the aphids samples captured in 2004.

AREA	SPECIES	N° SAMPLES	N° SAMPLES INFECTED
Revò - Alta Val di Non	*Dysaphis plantaginea*	15	1
	Aphis pomi	50	9
Denno - Cunevo - Campodenno	*Dysaphis devecta*	11	0
Val d'Adige Nord	*Dysaphis plantaginea*	30	3
	Aphis pomi	15	0
	Dysaphis devecta	8	1
	Rhopalosiphum insertum	4	0
TOTAL		133	14

3B.1.3 - 2005 collection

The insects were captured only in three areas of Val di Non.

D. plantaginea and *A. pomi* were present in all the three areas while *E. lanigerum* only in the Tuenno - Cles and Denno - Cunevo - Campodenno area. In this year 56 out of 655 aphid samples were positive by the PCR analysis (**Tab. 30**).

Tab. 30: analysis of the aphids samples captured in 2005.

AREA	SPECIES	N° SAMPLES	N° SAMPLES INFECTED
Revò - Alta Val di Non	*Dysaphis plantaginea*	71	2
	Aphis pomi	10	0
Tuenno - Cles	*Dysaphis plantaginea*	205	8
	Aphis pomi	256	29
	Eriosoma lanigerum	22	4
Denno - Cunevo - Campodenno	*Dysaphis plantaginea*	71	5
	Aphis pomi	10	0
	Eriosoma lanigerum	10	8
TOTAL		655	56

3B.1.4 - 2006 collection

In 2006 only 26 *D. plantaginea* and 16 *E. lanigerum* were captured in the Denno - Cunevo - Campodenno area to establish the presence and the quantity of phytoplasma in different parts of the insect, separately (head and body). All the samples were positive by PCR analysis (**Tab. 31**).

Furthermore four AP positive *C. picta* psyllids were employed as reference.

Tab. 31: analysis of the parts of the body of aphid and psyllid samples captured in 2006.

INSECT	SPECIES	PART OF INSECT	N° SAMPLES	N° SAMPLES INFECTED
Aphid	*Dysaphis plantaginea*	Head	26	26
		Body	26	26
	Eriosoma lanigerum	Head	16	16
		Body	16	16
Psyllid	*C. picta*	Head	4	4
		Body	4	4
TOTAL			46	46

3B.2 - QUANTITATIVE ANALYSIS

All the AP positive samples were analysed by quantitative PCR. Like the psyllids, the data are expressed in "n° of phytoplasma copies on one insect". Therefore it's important that the analysed samples are constituted by one insect only.

3B.2.1 - 2003 collection

The analysis carried out on 26 *D. plantaginea* and 83 *A. pomi* samples (**Tab. 32**).

Most of the AP positive insects come from Denno - Cunevo - Campodenno and Val d'Adige Nord areas.

Tab. 32: q-PCR analysis of the aphid samples captured in the year 2003.

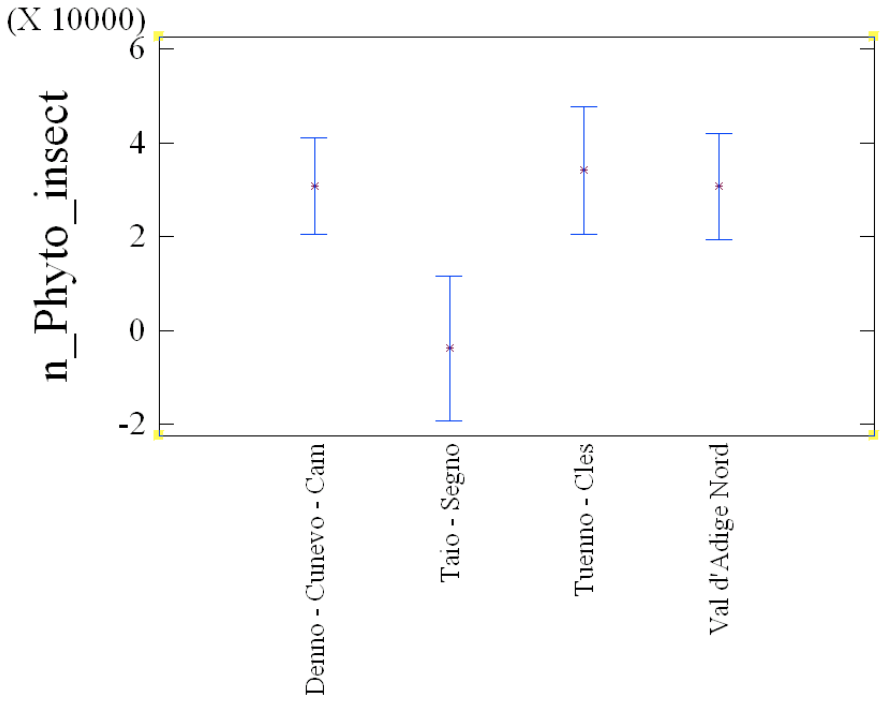
AREA	SPECIES	n° Phyto. / insect
Tuenno - Cles	*Dysaphis plantaginea*	351.538
	Dysaphis plantaginea	2.010
	Dysaphis plantaginea	937
	Dysaphis plantaginea	1.158
	Dysaphis plantaginea	2.388
Tuenno - Cles	*Aphis pomi*	2.504
	Aphis pomi	41.100
	Aphis pomi	3.423
	Aphis pomi	4.319
	Aphis pomi	3.823
	Aphis pomi	3.375
	Aphis pomi	5.794
Taio - Segno	*Dysaphis plantaginea*	21.772
	Dysaphis plantaginea	23.740
	Dysaphis plantaginea	565
	Dysaphis plantaginea	2.664
	Dysaphis plantaginea	67.840
	Dysaphis plantaginea	91.348
	Dysaphis plantaginea	4.522
	Dysaphis plantaginea	57.098
	Dysaphis plantaginea	2.035
	Dysaphis plantaginea	595
	Dysaphis plantaginea	3.181
	Dysaphis plantaginea	2.109
	Dysaphis plantaginea	9.211
	Dysaphis plantaginea	4.542
	Dysaphis plantaginea	3.634
Denno - Cunevo - Campodenno	*Dysaphis plantaginea*	25.250
	Dysaphis plantaginea	68.870
	Dysaphis plantaginea	69.201
	Dysaphis plantaginea	40.765
	Dysaphis plantaginea	65.587
	Dysaphis plantaginea	723
Denno - Cunevo - Campodenno	*Aphis pomi*	5.213
	Aphis pomi	6.392
	Aphis pomi	1.666
	Aphis pomi	45.885
	Aphis pomi	3.991
	Aphis pomi	815
	Aphis pomi	3.984
	Aphis pomi	37.623
	Aphis pomi	2.611
	Aphis pomi	2.046
	Aphis pomi	2.271
	Aphis pomi	5.939
	Aphis pomi	956
	Aphis pomi	7.253
	Aphis pomi	2.043
	Aphis pomi	10.010
	Aphis pomi	20.265
	Aphis pomi	21.387
	Aphis pomi	2.906
	Aphis pomi	5.818
	Aphis pomi	5.000
	Aphis pomi	17.654

Denno - Cunevo - Campodenno	*Aphis pomi*	5.812
	Aphis pomi	31.807
	Aphis pomi	5.495
	Aphis pomi	76.985
	Aphis pomi	11.921
	Aphis pomi	13.945
	Aphis pomi	4.061
	Aphis pomi	3.993
	Aphis pomi	59.035
Val d'Adige Nord	*Aphis pomi*	912
	Aphis pomi	2.070
	Aphis pomi	503
	Aphis pomi	4.779
	Aphis pomi	2.688
	Aphis pomi	1.197
	Aphis pomi	2.546
	Aphis pomi	648
	Aphis pomi	4.325
	Aphis pomi	873
	Aphis pomi	2.789
	Aphis pomi	20.775
	Aphis pomi	2.439
	Aphis pomi	1.433
	Aphis pomi	5.944
	Aphis pomi	15.896
	Aphis pomi	5.147
	Aphis pomi	125
	Aphis pomi	1.632
	Aphis pomi	2.222
	Aphis pomi	1.296
	Aphis pomi	1.390
	Aphis pomi	6.425
	Aphis pomi	2.851
	Aphis pomi	7.969
	Aphis pomi	55.789
	Aphis pomi	2.682
	Aphis pomi	27.366
	Aphis pomi	36.178
	Aphis pomi	15.673
	Aphis pomi	57.329
	Aphis pomi	754
	Aphis pomi	2.392
	Aphis pomi	1.835
	Aphis pomi	401
	Aphis pomi	1.772
	Aphis pomi	3.059
	Aphis pomi	2.492
	Aphis pomi	2.678
	Aphis pomi	2.892
	Aphis pomi	40.378
	Aphis pomi	2.708
	Aphis pomi	9.321
	Aphis pomi	2.158
	Aphis pomi	104.775

3B.2.1.1 - Areas

The concentration of pathogen within the insects collected in the Taio - Segno area is significantly different from that of other areas. The concentration of this area is lower (**Fig. 60**).

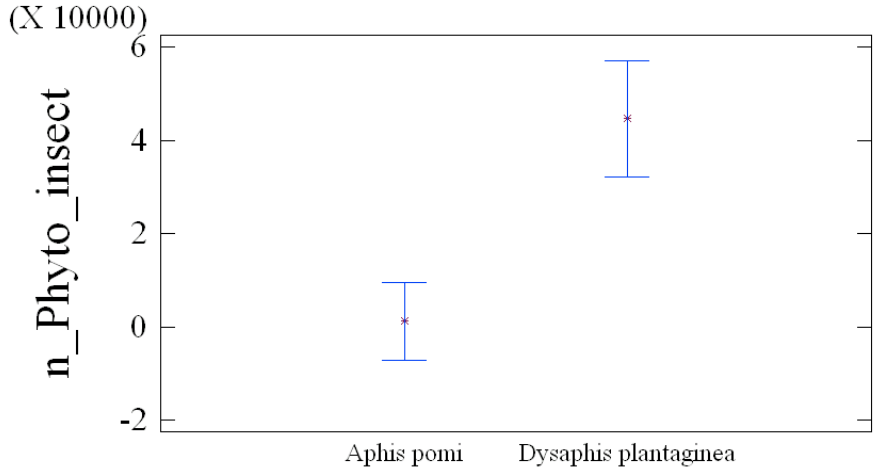
Fig. 60: comparison of phytoplasmal concentration in the areas in 2003.



3B.2.1.2 - Species

According to the analysis of variance there is a significant different of phytoplasmal concentration between the two species collected. In *D. plantaginea* the concentration is higher than that present in *A. pomi* (**Fig. 61**).

Fig. 61: comparison of phytoplasmal concentration between *A. pomi* and *D. plantaginea* collected in the year 2003.



3B.2.2 - 2004 collection

In this collection only 14 samples were analysed by q-PCR: 4 *D. plantaginea*, 9 *A. pomi* and one *D. devecta* sample (**Tab. 33**).

The samples come from the Revò - Alta Val di Non and Val d'Adige Nord areas.

Tab. 33: q-PCR analysis of the aphid samples captured in the year 2004.

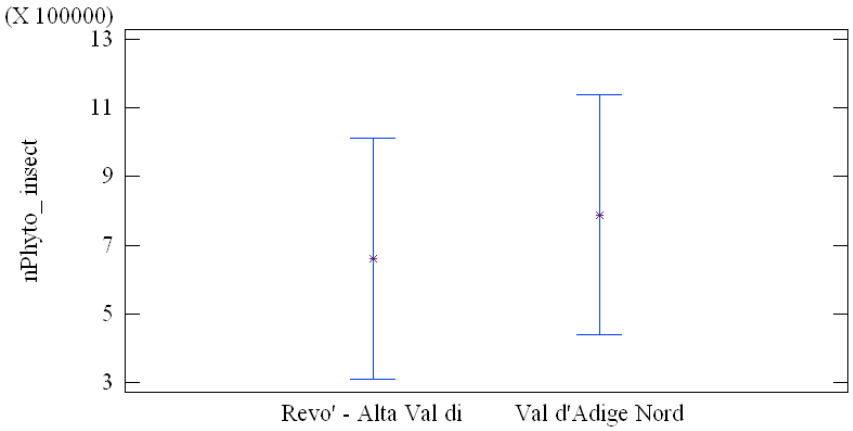
AREA	SPECIES	n° Phyto. / insect
Revo' - Alta Val di Non	*Dysaphis plantaginea*	235.542
Revo' - Alta Val di Non	*Aphis pomi*	222.418
	Aphis pomi	863.741
	Aphis pomi	351.959
	Aphis pomi	682.190
	Aphis pomi	163.568
	Aphis pomi	273.896
	Aphis pomi	332.614
	Aphis pomi	521.938
	Aphis pomi	200.017
Val d'Adige Nord	*Dysaphis plantaginea*	363.807
	Dysaphis plantaginea	774.909
	Dysaphis plantaginea	2.155.275
Val d'Adige Nord	*Dysaphis devecta*	1.527.049

3B.2.2.1 - Areas

There is no great difference of phytoplasmal concentration between the Revò - Alta Val di Non and Val d'Adige Nord area (the latter is slightly high) (**Fig. 62**).

In the Val d'Adige Nord area the concentration in 2004 is higher than that in 2003 (about 800.000 versus 30.000 copies of phytoplasma in one insect).

Fig. 62: comparison of phytoplasmal concentration in the areas in 2004.

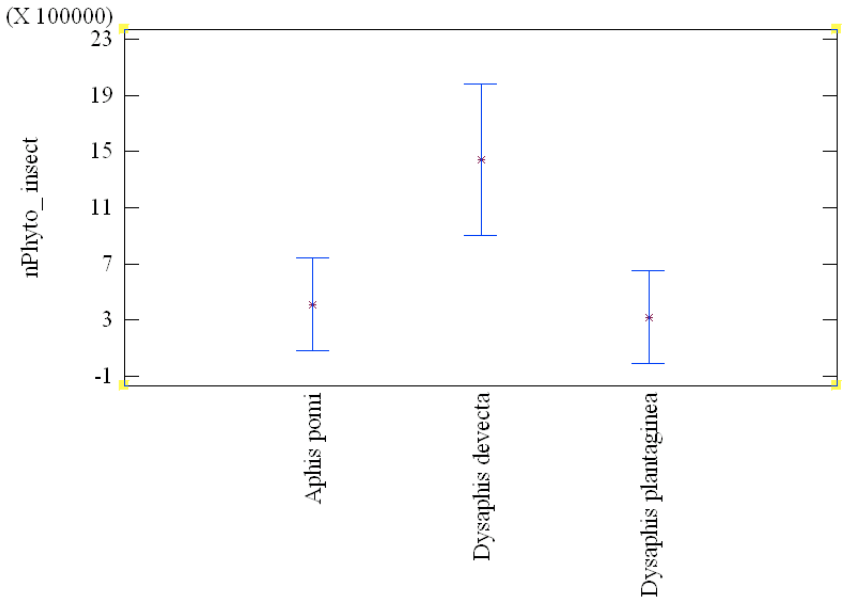


3B.2.2.2 - Species

According to the analysis of variance the phytoplasmal concentration is significantly different between *D. devecta* and the other species. *D. devecta* presents a higher concentration (about 1.500.000 on average) (**Fig. 63**).

The value of the *A. pomi* and *D. plantaginea* in 2004 is higher than that of 2003 (about 300.000 versus a few thousands of copies in one insect).

Fig. 63: comparison of phytoplasmal concentration present in the different species in 2004.



3B.2.3 - 2005 collection

The analysis carried out on 15 *D. plantaginea*, 29 *A. pomi* and 12 *E. lanigerum* samples (**Tab. 34**).

All the infected *A. pomi* come from the Tuenno - Cles area. The infected *D. plantaginea* come from all the collection areas.

Tab. 34: q-PCR analysis of the aphid samples captured in the year 2005.

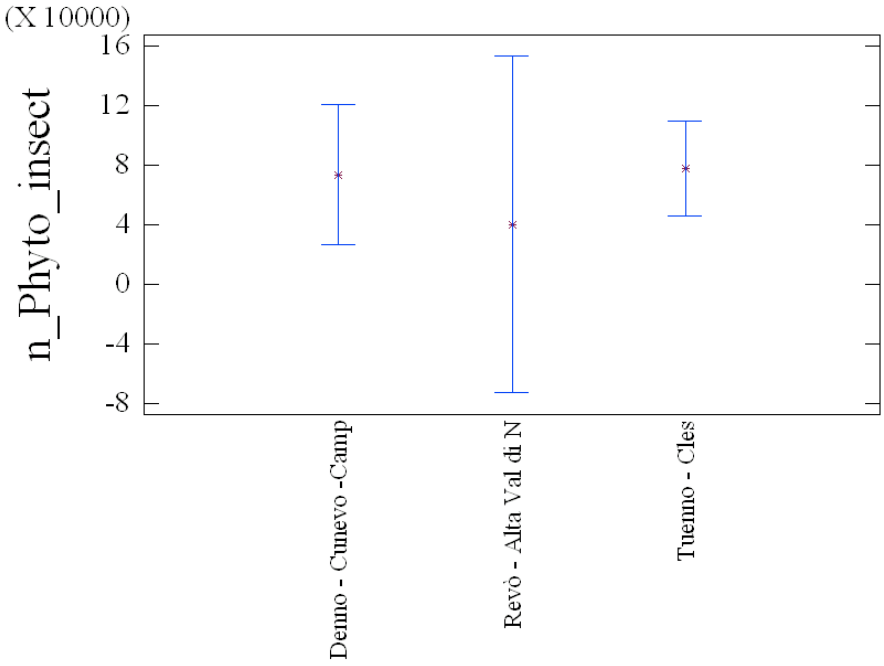
AREA	SPECIES	n° Phyto. / insect
Revò - Alta Val di Non	*Dysaphis plantaginea*	15.665
	Dysaphis plantaginea	43.999
Tuenno - Cles	*Dysaphis plantaginea*	12.052
	Dysaphis plantaginea	21.737
	Dysaphis plantaginea	112.162
	Dysaphis plantaginea	63.706
	Dysaphis plantaginea	77.099
	Dysaphis plantaginea	69.494
	Dysaphis plantaginea	100.467
	Dysaphis plantaginea	66.607
Tuenno - Cles	*Aphis pomi*	65.917
	Aphis pomi	65.336
	Aphis pomi	43.755
	Aphis pomi	38.259
	Aphis pomi	68.179
	Aphis pomi	86.380
	Aphis pomi	141.487

Tuenno - Cles	*Aphis pomi*	26.719
	Aphis pomi	62.303
	Aphis pomi	143.425
	Aphis pomi	97.466
	Aphis pomi	17.141
	Aphis pomi	373.555
	Aphis pomi	44.425
	Aphis pomi	80.596
	Aphis pomi	194.280
	Aphis pomi	123.296
	Aphis pomi	658.103
	Aphis pomi	48.996
	Aphis pomi	93.340
	Aphis pomi	103.407
	Aphis pomi	41.984
	Aphis pomi	328.249
	Aphis pomi	115.782
	Aphis pomi	19.881
	Aphis pomi	46.209
	Aphis pomi	30.234
	Aphis pomi	21.095
	Aphis pomi	359.534
Tuenno - Cles	*Eriosoma lanigerum*	101.995
	Eriosoma lanigerum	30.997
	Eriosoma lanigerum	41.726
	Eriosoma lanigerum	18.647
Denno - Cunevo - Campodenno	*Dysaphis plantaginea*	14.321
	Dysaphis plantaginea	102.705
	Dysaphis plantaginea	62.355
	Dysaphis plantaginea	139.804
	Dysaphis plantaginea	13.013
Denno - Cunevo - Campodenno	*Eriosoma lanigerum*	29.104
	Eriosoma lanigerum	79.663
	Eriosoma lanigerum	63.765
	Eriosoma lanigerum	10.966
	Eriosoma lanigerum	35.689
	Eriosoma lanigerum	12.069
	Eriosoma lanigerum	42.317
	Eriosoma lanigerum	27.105

3B.2.3.1 - Areas

The average concentration of phytoplasma is similar in all the three areas (**Fig. 64**).

Fig 64: comparison of phytoplasmal concentration in the areas in 2005.

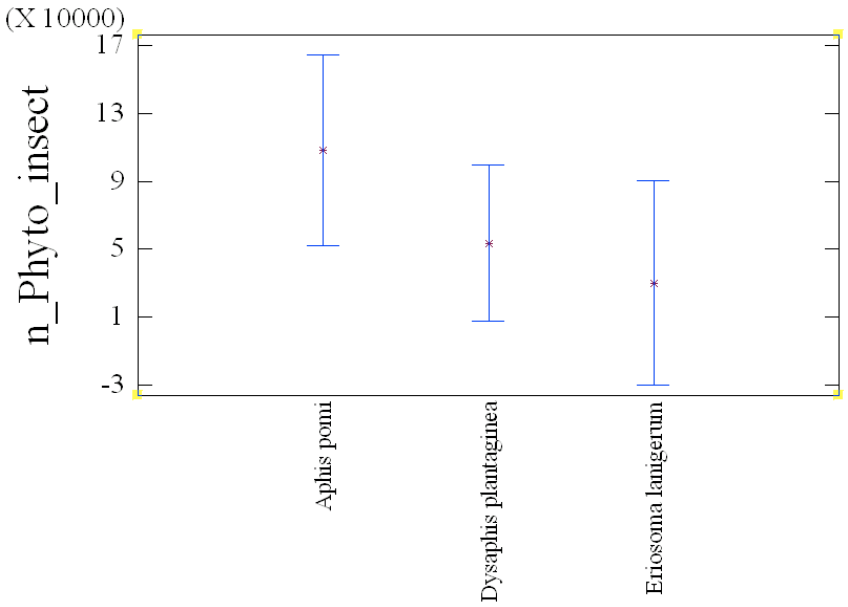


3B.2.3.2 - Species

The average phytoplasmal concentration in *A. pomi* is higher than that present in the other species. The value is lower in the *E. lanigerum* samples (**Fig. 65**).

The value of the average concentration in the *D. plantaginea* is declined to the value of the year 2003 (about 50.000 copies in one insect) while in *A. pomi* it decline to 110.000 copies about. It remains a high value (in 2003 there were about 1000 copies in one *A. pomi*).

Fig. 65: comparison of phytoplasmal concentration present into the different species in 2005.



3B.2.4 - 2006 collection

All the samples of the head and body of the insects were analysed by the q-PCR technique (**Tab. 35**).

In aphids *D. plantaginea* and *E. lanigerum* no samples with more than one million copies was observed while in the psyllids *C. picta* the concentration could exceed in some cases to half a billion of copies, particularly in the body.

In all the insects analysed the data that clearly emerges is a higher concentration of phytoplasma in the body than the head. Furthermore a higher difference in phytoplasma concentration was detected between the parts of the psyllid body as compared to the parts of the aphid body.

According to the analysis of variance there are not significant differences between the data of *D. plantaginea* and *E. lanigerum* but there is a significant difference between the aphids and *C. picta*. The concentration in the latter is higher than the two aphid species (**Fig. 66-67**).

Tab. 35: q-PCR analysis of the parts of the body of aphid and psyllid samples captured in the year 2006.

INSECT	SPECIES	n° Phyto. / Head	n° Phyto. / Body
Aphid	*Dysaphis plantaginea*	166.505	295.245
		172.662	301.585
		138.574	297.183
		94.300	259.111
		301.597	349.087
		226.829	352.282
		319.496	408.120
		232.001	479.100
		24.244	79.279
		35.526	120.671
		37.201	87.606
		37.999	66.339
		20.372	78.078
		16.714	59.267
		26.220	88.635
		26.615	110.128
		32.667	114.307
		45.287	112.975
		42.464	111.516
		33.591	110.152
		31.266	102.812
		23.256	95.595
		22.326	85.289
		29.845	98.713
		25.408	94.073
		42.589	133.954
Aphid	*Eriosoma lanigerum*	15.817	58.560
		28.998	75.730
		40.652	76.328
		28.909	75.489
		10.311	77.056
		40.432	73.948
		30.293	89.618
		243.058	465.249
		196.345	262.966
		8.042	274.731
		243.421	268.098
		232.400	288.084
		218.820	242.991
		229.255	261.674
		286.745	341.428
		38.771	81.751
Psyllid	*C. picta*	12.327.352	1.430.000.000
		3.103.855	455.000.000
		794.491	97.000.000
		30.823	2.103.599

Fig. 66: phytoplasmal concentration in head and body of aphids and psyllids. ■ *D. plantaginea* body; ■ *D. plantaginea* head; ▲ *E. lanigerum* body; ▲ *E. lanigerum* head; ○ *C. picta* body; ● C. picta head.

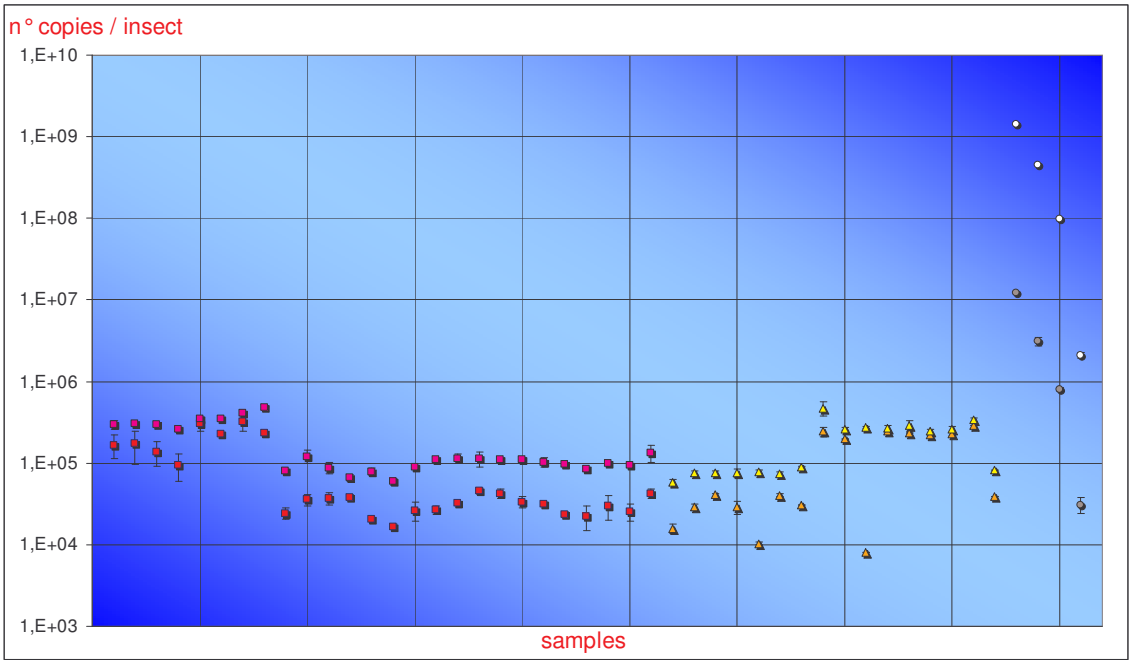
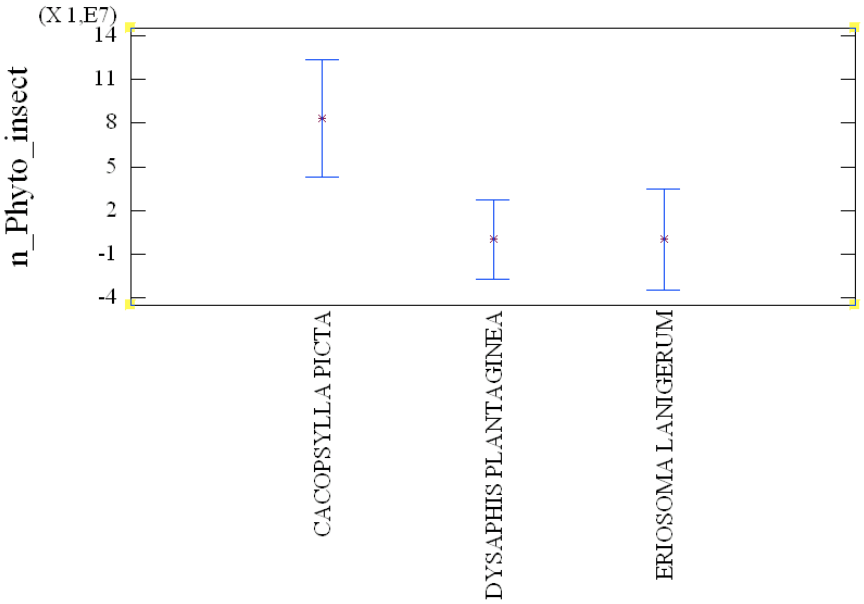


Fig. 67: total phytoplasmal concentration in the insect samples analysed by q-PCR in 2006.



4 - CONCLUSIONS

4.1 - POPULATION STRUCTURE OF AP IN TRENTINO

The Apple Proliferation disease caused by *Ca.* Phytoplasma mali, is largely diffused in Trentino. It has become a serious epidemic and an economic problem since ten years, in particular, in Val di Non and in Val di Sole where the cultivation of Apple orchards is intensive.

Since a few years three strains of phytoplasma have been defined (Jarausch *et al.*, 2000): AT1, AT2 e AP strains. They differentiate from each other by two point mutations. The distribution of these strains is noted in some orchards of France and Germany.

This research presented an initial study on the population dynamics of *Ca*. Phytoplasma mali. The kind of distribution of the strains and its eventual change in time was determined. The correlation between the presence of the strain and some parameters of the plants, insect vectors and the territory was researched.

The distribution of the strains of *Ca.* Phytoplasma mali in Trentino is immediately appears particular. In France and Germany AP strain is known to be widespread while the AT1 and the AT2 strains are less frequent (Jarausch *et al.*, 2000; Jarausch *et al.*, 2004). On the contrary, the situation in Trentino is completely the opposite. The AT2 strain is more widespread while the presence of the AT1 strain can be noted only in some areas of South Trentino. The presence of the AP strain is rare.

Two different situations emerged from the analysis of the strain distribution in the areas in 2002.

There is a high rate of diseased plants in the five areas of North-West Trentino, located in Val di Sole and Val di Non, where intensive apple orchards are grown. This entire territory can be considered a high infection zone. In these areas the presence of AT2 strain is absolutely predominant.

In the other five areas of Central and South Trentino apple growing is less widespread and the pathogen is less present. These areas are located in a low infection zone. Here the frequency of AT1 strain was higher and is more than 50% in the Val d'Adige Sud area. From North to

South the presence of the AT1 strain increased: there was a gradient distribution of the AT2 and the AT1 strains. The AP strain is completely absent.

In 2004 the situation was slightly modified. While in the high infection zone the distribution is stationary, in the low infection zone the percentage of AT1 strain is proportionally reduced to half its value.

A high correlation between the strain and the geographic area is evident and it seems that as time goes by the AT2 strain is spreading more also in the South of Trentino.

The increase in the collection does not show a difference in the distribution of the strain. The strain distribution in the micro-area roughly reflects the situation present in the areas where they are located. The novelty is the presence of one sample of AP strain in Bonifica - Rumo microarea in Revò - Alta Val di Non area and in Focus Non - Sporminore micro-area in Denno - Cunevo - Campodenno area.

In 2005 the region of Nanno represents the actual situation in Val di Non. AT2 strain is widespread and there are a few samples infected with the AP strain. The AT1 strain is now absent. This might be because of its gradual decrease each year.

The model region of Piovi also shows the distribution of the strains in the areas of the low infection zone. The AP is completely absent and a low percentage of the AT1 strain is present. Also in this case this might be because of its gradual decrease each year. This region is to the North of Trentino, where there is a lower concentration of the AT1 strain in the low infection zone. It should be interesting to notice another model region to the South of Trentino where, probably in the future, there will not be any AP strain and the AT1 strain will become more frequent.

It would be interesting to monitor the diffusion of the AT1 and the AP strains in the respective regions to understand if their distribution could be an instrument to verify the progress of the disease.

It appears that the distribution of the strain is not in relation to the age of plant and neither in relation to the rootstock.

The psyllid *C. picta* overwintering was found more infected than *C. melanoneura*. One presumes that the *C. picta* is already infected when it comes back to the apple trees after winter, while *C. melanoneura* acquires the phytoplasma later. The low presence of phytoplasma in insects of the new generation could be connected with the time required for the incubation of the phytoplasma and the gathering of the insects.

During the last few years the percentage of infected insects has greatly decreased in all the species.

The number of infected insects is low and the population of phytoplasma can be analyzed taking into consideration the two infection zones only. However, in the same infection zone the analysis of the variability of the population of the phytoplasma in the psyllids confirms the situation present in the plant samples, both in 2002 and 2004, and also the correlation between the strain and the geographic area.

The distribution of the strain in the psyllid species and in those zones is the same. The above is obvious because *C. picta* is mainly present in the North-West of Trentino and *C. melanoneura* lives mainly in the low infection zone.

There should be an increase in the collection of insects in the future to complete the missing data in certain areas. In the last few years the number of psyllids is greatly reduced. Hence it will be difficult to have more information on regarding this.

According to data collected, the concentration of phytoplasma in the samples when they are infected by AT1 strain, is higher than when they are infected by the AT2 or AP strain. However, the number of AT1 infected samples is very low. The number of this data ought to increase to verify whether the concentration in the samples is related to the kind of strain.

The most interesting data given by the quantitative analysis of the model regions is that concerning the rootstocks and the age of plants.

The samples with M9 rootstock presented a higher concentration. Statistically, the value in these samples were significant different from the Franco and M11 rootstock.

It seems that in Nanno the concentration of the pathogen is indirectly proportional to the age of the plant. The plants aged 9 years show a high concentration of the pathogen. It seems that the situation is inverted in Piovi: the concentration increases with the age. But in Nanno the interval of the plants analysed was 61 years (from 9 to 70 years) while in Piovi the interval was 18 years only (from 0 to 18 years). In future the older plants coming from Piovi must be analysed to verify whether in these two model regions the trend is different or whether a major concentration is present in the relatively younger plants.

The quantitative data drawn of the psyllids does not furnish the necessary information.

It would be very important to deepen the quantitative data linked to the strain. If the concentration is connected with the intensity of disease symptomatology it should possible to choose the best material for the orchards (the best rootstock). Again, if there should be a relation between strain, concentration and symptomatology it should somehow associate the presence of the symptom with the strain.

The study on the population dynamics of *Ca*. Phytoplasma mali has highlighted the situation present in Trentino. For the moment the data does not furnish much information to conquer

the disease. This data could have an important role if, in the future, the *in vitro* analysis will be able to evaluate the virulence of the different strains.
It would be interesting to relate this data with the recent information furnished by the genetic variability analysis on some regions of the phytoplasma genome that can be associated with the mechanism of the infection.

4.2 - AP TRANSMISSION CAPACITY OF APHIDS IN TRENTINO

C. picta and *C. melanoneura* are well known vectors of *Ca.* Phytoplasma mali. In the Trentino region only *C. picta* has been proved to be associated with AP spreading (Frisinghelli *et al*., 2000), but at present its rare presence in orchards does not explain the virulence of the disease.
Therefore a first research was made to investigate the existence of further vectors by checking the presence of the pathogen in insect species and subsequently evaluating their capability of transmission.
The research is focused on aphids as they are widely diffused in the cultivation of apple trees. The aphids are noted to be vectors of virus but they are never found to be vectors of phytoplasma. Hence, it was a great surprise to find the first samples of *D. plantaginea* infected.
For three years their presence was monitored in the different areas of Val di Non and Val d'Adige, capturing diverse species. *D. plantaginea*, *A. pomi*, *E. lanigerum* e *D. devecta* were PCR positive. The first two species were captured every year.
In the initial research the presence of the pathogen was verified and in succession the concentration of the pathogen in the insect was determined. The vector *C. picta* noted in Trentino, was taken as reference.
After three years of research it was confirmed that the phytoplasma was effectively present in the aphids. It not yet clear whether there is a relationship between the concentration of the pathogen and the aphid species.
D. plantaginea collected in June 2003 showed a higher concentration of pathogen in respect to *A. pomi* collected in August of the same year.
In the year 2004 the concentration of the pathogen in the aphids was 10 times higher than that of 2003. One sample of *D. devecta* captured in Spring was infected and presented more than

one million copies of phytoplasma. In future it is necessary to capture this specie of aphids, in late Spring, in Val di Non and Val d'Adige. The analysis of these samples would verify the progress of the concentration of the phytoplasma in the *D. devecta*.

In the year 2005 the values of the average concentration declined to the value of the year 2003. The *A. pomi* samples captured in July and August showed a higher concentration of phytoplasma while the values of the pathogen in *E. lanigerum* samples, captured mainly in June, were lower.

The time of the sample collection, also called the harvest period, can affect the concentration of the pathogen in the insect a lot because the phytoplasma in evenly distributed in the plant only from July onwards. If the aphid captured in June fed in a part of the plant where the phytoplasma is scarse, the concentration of the pathogen within it would probably be scarce too. The period of the capture will therefore be an important parameter to be taken into consideration to compare data.

The area of capture has not provided much information.

In 2003 a low concentration of the pathogen was noted in the Taio - Segno area. In this area the insects were captured in June while in the other areas the samples were also captured in August. In 2004 there was not much of a difference in the concentration of the phytoplasma between the different areas. The situation was similar in the year 2005.

There is a big difference in the concentration of the phytoplasma in psyllid *C. picta* overwintering in respect to that of the phytoplasma in the aphids. The concentration of phytoplasma is much more elevated (100 times) in the vector than in the aphid.

It is noted that the psyllid *C. picta* overwintering probably returns to the apple trees after winter bringing along with it a good concentration of phytoplasma. The psyllid had the possibility to get infected the previous year. It would be better to compare the aphid with the young stage of *C. picta* or with its new generation.

The aphid belongs to the new generation and reproduces rapidly. During its short life time, the insect remains on the plant for a brief period (*A. pomi* excepted). One should know for how long the aphid analysed fed on the infected plant and the time in which the pathogen was acquired.

The two insects should be captured in the same period, after July, when the diffusion of the phytoplasma in the plant is homogeneous. The insects should stay on the plant for the same identical period.

Regarding the anatomy of the two insects there still isn't any information on the position of the salivary glands where the phytoplasma multiplies. The psyllid has its head, chest and

abdomen well distinguished hence it is simple to cut off its head correctly. The same cannot be done in the case of the aphid as its structure is different.

As result of this initial work on the aphids it can be seen that phytoplasma is present in these insects. The presence of the phytoplasma in an aphid had not been proved before this. These insects acquire a small quantity of phytoplasma (don't ever exceed one million copies). This quantity is much smaller than that present in *C. picta* (even more than half a billion).

In the Istituto Agrario di San Michele all'Adige, during the same period as the above experiments, transmission trials were carried out on these insects to verify if the insect could be a vector of *Ca*. Phytoplasma mali. The results were negative. The molecular data collected seems to confirm this. The value of the low concentration should be verified. The concentration of the pathogen in the aphid can be compared to that of *C. melanoneura*, in similar conditions. This psyllid has transmission efficiency inferior to that of *C. picta*. One could verify if the concentration of the pathogen in the insect is in relation to its transmission efficiency.

This work could be prepared in the greenhouse along with the transmission trial. Taking into consideration the same period of capture, the same time of acquisition, the same position of the insects on the plant and the same distribution and concentration of phytoplasma in the plant; and with major information on the anatomy of aphids and psyllids, one could probably verify the difference between the species of aphids, the difference between the aphids and the psyllids and what exactly their role is in the transmission of the Apple proliferation disease.

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