

## Rooting of Healthy and Cvc-Affected 'Valência' Sweet Orange Stem Cuttings, through the Use of Plant Regulators

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### ABSTRACT

*Citrus variegated chlorosis (CVC) is a disease caused by Xylella fastidiosa. Using different concentrations of plant regulators, such as auxins (indole-3-butyric acid) and gibberellic acid biosynthesis-inhibitor (paclobutrazol), physiological rooting capacity of healthy and CVC-affected stem cuttings were evaluated in order to investigate the importance of plant hormone imbalance and xylem occlusion in plants with CVC. The percentages of dead, alive and rooted cuttings, cuttings with callus and mean number of roots per cuttings did not show statistical differences in response to the distinct concentrations of synthetic plant regulators. There were differences only between healthy and CVC-affected cuttings. This showed the importance of xylem occlusion and diffusive disturbances in diseased plants, in relation to root initiation capacity and hormonal translocation in the plant tissue.*

**Key words:** IBA, Paclobutrazol, *Xylella fastidiosa*, root initiation, *Citrus sinensis*

### INTRODUCTION

CVC has been observed in São Paulo and Minas Gerais States, Brazil, since 1987 (Rossetti et al., 1990), and it has been causing a yearly loss of about 100 million U.S. dollars to the Brazilian citrus industry, just in the São Paulo State (Laranjeira, 1997). This disease is caused by the bacterium, *Xylella fastidiosa* (Leite Junior and Leite, 1991; Lee et al., 1993; Chang et al., 1993; Hartung et al., 1994). There are many important diseases caused by other strains of *X. fastidiosa*, including Pierce's disease of grapevine (PD) and others (Purcell and Hopkins, 1996). Symptoms include conspicuous variegations on older leaves, with chlorotic areas on the upper side and corresponding light brown lesions on the lower side. Affected fruits become smaller, hardened,

with higher sugar content (Rossetti et al., 1990; Rossetti and De Negri, 1990; Laranjeira and Palazzo, 1999).

Potassium deficiency is common in plants affected for a period of time (Malavolta et al., 1993). CVC-affected plants have leaves with water deficiency symptoms, associated with significant decreases in the net photosynthesis and transpiration rates (Machado et al., 1994). Goodwin et al. (1988) related PD with low leaf water potential and increased xylem flow resistance. Machado et al. (1994) suggested that these processes may be due to stomatal dysfunction, increased water resistance through xylem and/or decreased water uptake making these plants more sensitive to water deficiency.

There is strong evidence that *X. fastidiosa* plugs xylem vessels (Rossetti et al., 1990). However,

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Hopkins (1989) suggested two other hypotheses for the pathogenicity: bacterial phytotoxins and plant hormone imbalance, which could have some physiological interactions with the root initiation capacity in stem cuttings. Exogenous application of gibberellic acid in peach trees infected with *X. fastidiosa* promoted partial remission of symptoms (French and Stassi, 1978).

Exogenous application of IAA and kinetin in *Vitis rotundifolia* cv. Carlos, moderately resistant to PD, prevented symptoms development, as well as bacteria accumulation in leaves. When these treatments were applied in *Vitis vinifera* cv. Carignane, highly susceptible to PD, the plant regulators did not prevent symptoms (Hopkins, 1985). None of these plant regulators prevented the *in vitro* bacterial growth, suggesting that its effect must have been in the host. This partial remission of symptoms in *X. fastidiosa*-infected plants treated with auxin and gibberellins could be suggested as plant regulator imbalance.

There are many factors influencing root initiation in cuttings, such as source of stem cuttings, time of the day for cutting preparation, seasonal time, rooting media and plant regulators used. Natural auxins, such as indole-3-acetic acid (IAA) and synthetic ones, such as indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) stimulate the production of adventitious roots in stem and leaf cuttings. The response, however, is not universal. Cuttings of some difficult-to-root species still root poorly after treatment with auxins (Hartmann et al., 2002). IBA is also a naturally occurring substance in plants (Ludwig-Muller and Epstein, 1994). In apple, when IBA is applied to stem cuttings, it is, in part, converted to IAA (Vander Krieken et al., 1992). IBA may also enhance rooting via increased internal-free IBA, or may synergistically modify the action or endogenous biosynthesis of IAA (Hartmann et al., 2002).

It has been confirmed that auxin is required for initiation of adventitious roots in stems, and indeed, it has been shown that divisions of the first root initial cells are dependent upon either applied or endogenous auxin (Thimann and Poutasse, 1941; Haissig, 1972; Tillburg, 1974; Strömquist and Hansen, 1980; Maldiney et al., 1986). Synthetic substances have been used, together with auxins, in order to enhance the rooting capacity of stem cuttings from many species. Shoot growth retardants, such as paclobutrazol, a gibberellin (GA) biosynthesis-inhibitor, antagonizes GA biosynthesis or activity and reduces shoot growth,

resulting in less competition and consequently more assimilates are available for rooting at cutting bases, which helps to promote rooting (Hartmann et al., 2002).

Despite of the plant hormone imbalance hypothesis for *X. fastidiosa* pathogenicity, little is known about this.

This work examined the physiological rooting capacity of cuttings from CVC-affected plants compared to healthy ones, in order to investigate the hypothesis of plant hormone imbalance in plants with CVC and its interactions with exogenous applied plant regulators.

## MATERIAL AND METHODS

Stem cuttings from healthy and CVC-affected plants were collected from sweet orange (*Citrus sinensis* L. Osbeck cv. Valência) commercial groves (Pratânia-SP, Brazil, 22° 44' south latitude and 48° 34' west Greenwich longitude), with 9 and 10 yr-old plants, respectively. The bacterial presence was confirmed through polymerase chain reaction (PCR) test, specific for *X. fastidiosa* from CVC-strain, as described by Pooler and Hartung (1995). Mature terminal growth stems were cut between 7:00 and 9:00 a.m. on August 25<sup>th</sup>, 2000, and it was put in moist plastic bags and taken to the lab. Stems were separated into healthy and CVC-affected material and cuttings were made with 15-20 cm long, 0.5 cm in diameter and lower two or three leaves removed. Next, they were submitted to a previous bath with Benomyl solution (1 gL<sup>-1</sup>), for 15 min.

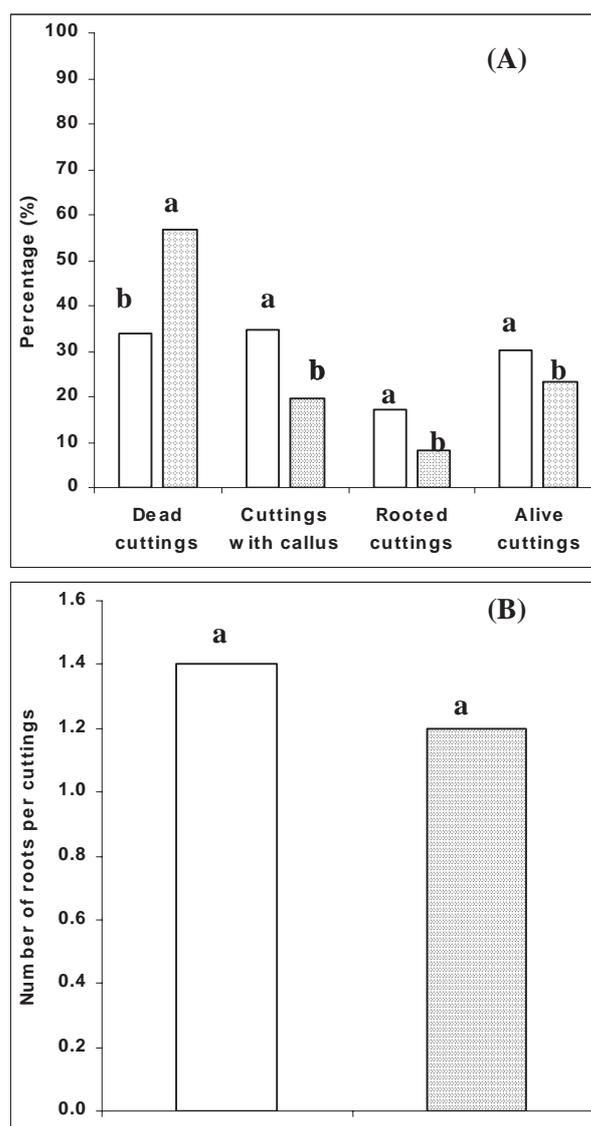
IBA (P.A.) was used as a source of auxin with concentrations of 2000 and 5000 mgL<sup>-1</sup>. Paclobutrazol (PBZ) (1-chlorophenyl-4,4-dimethyl-2-(1,2,4-triazol-1-yl) penta-3-ol) (commercially known as PP333 or Bonzi) was used at the concentration of 2000 mgL<sup>-1</sup>. The combinations of these two plant regulators used characterized the treatments (T), which were: T1: water (control), T2: IBA 5000 mgL<sup>-1</sup>; T3: IBA 2000 mgL<sup>-1</sup>; T4: PBZ 2000 mgL<sup>-1</sup>; T5: IBA 5000 mgL<sup>-1</sup> + PBZ 2000 mgL<sup>-1</sup> and T6: IBA 2000 mgL<sup>-1</sup> + PBZ 2000 mgL<sup>-1</sup>. The auxin solutions used were prepared with alcohol and the PBZ solutions with distilled water.

Isopor trays (288 cells) with rooting media made of carbonized rice husk were moistened in the intermittent fog system for 24 h. The concentrated

solution dip method ("quick dip" - 5 sec.) was used to treat the base of cuttings, which were stuck into the trays that were taken into the fog system. The air temperature in the fog system was kept at  $25 \pm 2^\circ\text{C}$  and water sprays were applied each 15 min. Disease control was done by spraying  $1 \text{ gL}^{-1}$  Benomyl +  $1 \text{ gL}^{-1}$  Captan solution, every 15 days. After these sprayings, the fog system was kept off for 1 h. Ninety days after treatments were applied, the cuttings were collected and following variables were measured: percentages of dead, alive and

rooted cuttings, cuttings with callus and number of roots per cuttings.

This study was conducted in a completely randomized experimental design, with 2 levels of health (CVC-affected and healthy cuttings) and 6 treatments (2 x 6) with 3 replications and 20 cuttings per replication. Mean values were subjected to one-way factorial analysis of variance and compared by Tukey's test at 5% level of probability.



**Figure 1** - Percentages of dead, rooted and alive cuttings, and cuttings with callus (A) and number of roots per cuttings (B) of healthy and CVC-affected cuttings of 'Valência' sweet orange plants, after 90 days in the rooting media, under intermitent fog system.

The same letters are not significantly different from each other by Tukey's test ( $p \leq 0.05$ ) Empty columns represent the healthy cuttings and full columns, the CVC-affected ones. Mean values were transformed by arc sine of  $(x + 0.5)$  square root.

**Table 1** - Mean values of rooting variables of healthy and CVC-affected cuttings of 'Valência' sweet orange plants, after 90 days in the rooting media, under intermittent fog system.

Treatments	Dead Cuttings	Cuttings with callus (%)	Rooted cuttings	Alive cuttings <sup>1</sup>	Number of roots per cuttings Mean values
Water (control)	41.8 a	31.8 a	9.7 a	27.7 a	1.1 ab
IBA 5000 mgL <sup>-1</sup>	45.8 a	22.0 a	15.3 a	29.7 a	1.6 ab
IBA 2000 mgL <sup>-1</sup>	47.1 a	31.1 a	15.4 a	19.3 a	1.3 ab
PBZ <sup>2</sup> (2000 mgL <sup>-1</sup> )	47.7 a	24.2 a	5.6 a	30.4 a	0.8 b
IBA 5000 mgL <sup>-1</sup> + PBZ	37.4 a	34.4 a	19.6 a	22.2 a	1.9 a
IBA 2000 mgL <sup>-1</sup> + PBZ	52.3 a	19.7 a	10.6 a	24.2 a	1.2 ab
C.V. <sup>3</sup> (%)	19.5	38.4	63.4	32.9	46.3

<sup>3</sup> 3 replications and 20 cuttings per replication were used. Means were transformed by arc sine of  $(x + 0.5)$  square root.

The same letters in columns are not significantly different from each other by Tukey's test ( $p \leq 0.05$ ).

<sup>1</sup> unrooted and without callus, but kept alive; <sup>2</sup> Paclobutrazol; <sup>3</sup> Coefficient of variation.

## RESULTS AND DISCUSSION

PCR tests confirmed the bacterial presence in CVC-affected plants while the non-symptomatic plants from the healthy orchard were negative. Differences among variables were observed between healthy and diseased cuttings (Figs 1A and 1B). However, there were no differences among variables in response to plant regulators and its different concentrations applied. Thus there was no significant statistical interaction between the level-of-health factor and the treatment factor for the very measured variables (Table 1).

Healthy cuttings presented higher percentage of cuttings with callus, rooted and alive cuttings, when compared to CVC-affected ones, and the number of dead cuttings was higher in diseased cuttings than in the healthy ones (Fig. 1A). However, the number of roots per cuttings was not different between these two groups (Fig. 1B). This variable was, actually, measured in rooted cuttings, after root cells had already been initiated and formed. This suggested that despite of the higher difficulty for diseased cuttings to survive and initiate rooting (Fig. 1A), the auxin or the possible hormonal imbalance in CVC-affected rooted cuttings, would be so important as for the healthy cuttings. Hence, the number of cells that differentiated into adventitious roots under hormonal balance action was the same in both healthy or CVC-affected cuttings.

CVC-affected plants presented about 57% of dead cuttings, whereas the healthy ones showed only 34% (Fig. 1A), indicating that the surviving capacity was independent on a possible plant hormone imbalance, since no statistical difference was observed between the level-of-health factor

and distinct concentrations of plant regulators applied. It could be more related to the dysfunction in the water conducting system, supported by the lower leaf water potential and stomatal conductances of sweet-orange plants affected by CVC (Machado et al., 1994, Gomes et al 2003a; Habermann et al., 2003a; Habermann, 2004).

Avocado cuttings of a difficult-to-root cultivar shed their leaves and died, which did not happen with rooted cuttings (Reuveni and Raviv, 1981). Since *X. fastidiosa* block xylem vessels (Rossetti et al., 1990), CVC-affected cuttings might have had more difficulty to sustain the transpiration "pull", which eventually provided water to the mesophyll. Then, with less water supply, leaves might have faced drought, what maybe contributed to the leaf shed and, consequently, to the death of cuttings.

Supporting this idea, Hartmann et al. (2002) emphasized the importance of taking cuttings early in the morning in order to avoid water stress and have the material in turgid condition. Pea cuttings showed reduced rooting when the cuttings were taken from stock plants presenting water deficit (Rajagopal and Andersen, 1980). Besides, unrooted cuttings are particularly vulnerable to water stress, since rehydration of the tissue is very difficult without a root system. But while presence of leaves can be important for rooting, leaf retention is more a consequence of rooting than a direct cause of it, and it may be related to the starch translocation from leaves to the base of cuttings (Ono and Rodrigues, 1996).

The biochemical transformation of starch into soluble sugars through auxin action in leaves, stems and roots (Alexander, 1938) is important and it is implicated in the rooting capacity of

cuttings. However, further than xylem blockage, CVC-affected plants have low net photosynthesis rates (Machado et al., 1994; Medina, 2002; Gomes et al. 2003b; Habermann et al., 2003a; Ribeiro et al., 2003; Habermann, 2004). There is also evidence of decreased rubisco enzyme efficiency (carboxylation efficiency) in CVC-affected plants (Habermann et al., 2003b), that is, the photosynthetic metabolism is affected as well, what indicates a lower starch production, accumulation and translocation from leaves to the base of cuttings. In fact, Queiroz-Voltan and Paradella Filho (1999) observed anatomical alterations in leaves of CVC-affected plants. These authors observed abnormal cellular divisions in the mesophyll and reduced number of chloroplasts. Thus, the so important starch for rooting of cuttings could be reduced, which explain the low rooted cuttings from diseased plants (Fig. 1A). Actually, alive, with callus and rooted CVC-affected cuttings represented those ones that did not shed their leaves. Therefore, the progression alive, with callus and rooted cuttings from CVC-affected plants expresses the auxin action over the differentiation process. But the lower starch production, accumulation and translocation from leaves to the base of CVC-affected cuttings could have slower the root differentiation.

*X. fastidiosa* promote the uptake of iron and possibly other transition metal ions, contributing to the typical symptoms of leaf variegation (Simpson et al., 2000). Gomes et al. (2003a) working with three cycles of water deficit in healthy and CVC-affected 'Pêra' sweet orange plants found low IAA concentrations in leaves of diseased plants of the first cycle, but no difference in the third one, when plants were physiologically more damaged by CVC. These authors related this to the fact that orange trees with CVC generally have low zinc contents in the leaves (Malavolta et al., 1993). Zinc is an essential element for tryptophan synthesis, one of the auxin precursors (Taiz, 2002a). Gomes et al. (2003a) suggested that the bacteria decreased, somehow, the zinc content in the plant, causing less IAA synthesis. However, Medina (2002) worked with the same plants of Gomes et al. (2003a) and found no difference in the leaf zinc contents between healthy and CVC-affected plants.

CVC-affected 'Valência' sweet orange plants presented a difficult to interpret oscillation in the leaf IAA, GA<sub>1</sub>, ethylen and zeatin contents (Habermann, 2004). In this work, the author also

studied the leaf gas exchange and water relations of plants with CVC and concluded that the bacterial colonization of xylem vessels, causing plant water imbalance and photosynthetic disturbances, was more important than a possible hormonal imbalance. On the other hand, the non-responsive concentrations of plant regulators used could have been below the effective doses. However, Rossal and Kersten (1997) showed that the highest percentage of formed callus in 'Valência' sweet orange cuttings was obtained with 4500 mgL<sup>-1</sup> of IBA, and this percentage started falling with higher concentrations. Hence, healthy plants presenting less than 20% of rooted cuttings (Fig. 1A) was in agreement with Platt and Opitz (1973), Castro and Kersten (1996) and Rossal and Kersten (1997), who described sweet orange cuttings as difficult-to-root ones.

The highest auxin concentration in plant tissues generally occurs during spring and summer (Hartmann et al., 2002; Taiz, 2002a). There are many reports suggesting the fall and/or winter not as the best seasons for rooting of cuttings (Ono and Rodrigues, 1996). Metabolical changes in cambium cells and plant hormone (im)balance may occur during these seasons (Hartmann, et al., 2002). This fact may have contributed to the low percentage of healthy rooted cuttings.

When plants face low temperatures and short photoperiod in winter, there are decrease in endogenous gibberellic acid and increase in abscisic acid (ABA), which lead to bud and seed dormancy (Taiz, 2002b). Actually it involves many other endogenous compounds, being more associated with a hormone balance. Adjustment of plant hormones concentrations were also reported during flowering and vegetative growth of grapevines (Niimi and Torikata, 1978). Between winter and spring, the increase of temperature and mainly the starting of longer photoperiods can be, somehow, perceived by phytochromes, and there is a complex biochemical signal system that eventually ends up with increasing of GA and decreasing of ABA (Taiz, 2002c).

Classic experiments with cuttings have shown that the initiation and growth of adventitious roots inhibit flowering in the shoot (Taiz, 2002c). Flowering induction could inhibit the adventitious root initiation and growth. Thus, it was expected that GA biosynthesis inhibitors could act positively on root initiation. But as with IBA concentrations used, it could have been below the effective doses, too. Despite this positive

involvement of GA with flowering, exogenous GA application could inhibit citrus flowering during flowering induction period (Monselise and Halevi, 1964; Davenport, 1990). In fact, the season chosen for this experiment could have coincided with the flowering induction period, since bloom occurred in early September.

Thus, 8.8% lower CVC-affected rooted cuttings (Fig. 1A) might be caused by the bacterial xylem occlusion and the lower starch production, accumulation and translocation, and not a possible hormonal imbalance. In other words, since sweet orange cuttings are known as difficult-to-root ones (even because of lignification and suberization of cuttings), and if auxin is one of the most important factor that affects adventitious root formation (Hartmann et al., 2002), on the other hand, it is still not clear if CVC-affected plants are IAA-deficient.

Then, it would be interesting to continue the plant hormone imbalance hypothesis in CVC-affected plants, including studies in other seasons and other factors involving adventitious root formation.

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## RESUMO

Clorose variegada dos citros (CVC) é uma doença causada por *Xylella fastidiosa*, podendo determinar oclusão do xilema e desbalanço hormonal, o que por fim está relacionado ao processo de iniciação radicial em estacas. Usando diferentes concentrações de fitorreguladores, como auxinas (ácido 3-indol butírico) e inibidores da biossíntese de ácido giberélico (paclobutrazol), que são promotores do enraizamento de estacas, verificou-se a capacidade fisiológica de enraizamento de estacas sadias e com CVC, a fim de investigar a importância do desbalanço hormonal e oclusão do xilema em plantas doentes. As porcentagens de estacas mortas, vivas, enraizadas e com calo e o número médio de raízes por estaca não mostraram diferenças estatísticas em resposta às diferentes concentrações dos

reguladores vegetais sintéticos. Houve diferenças apenas entre estacas sadias e doentes. Isto aponta a importância da oclusão do xilema e distúrbios difusivos em plantas doentes, em relação à capacidade de iniciação radicial e à translocação hormonal no tecido vegetal.

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