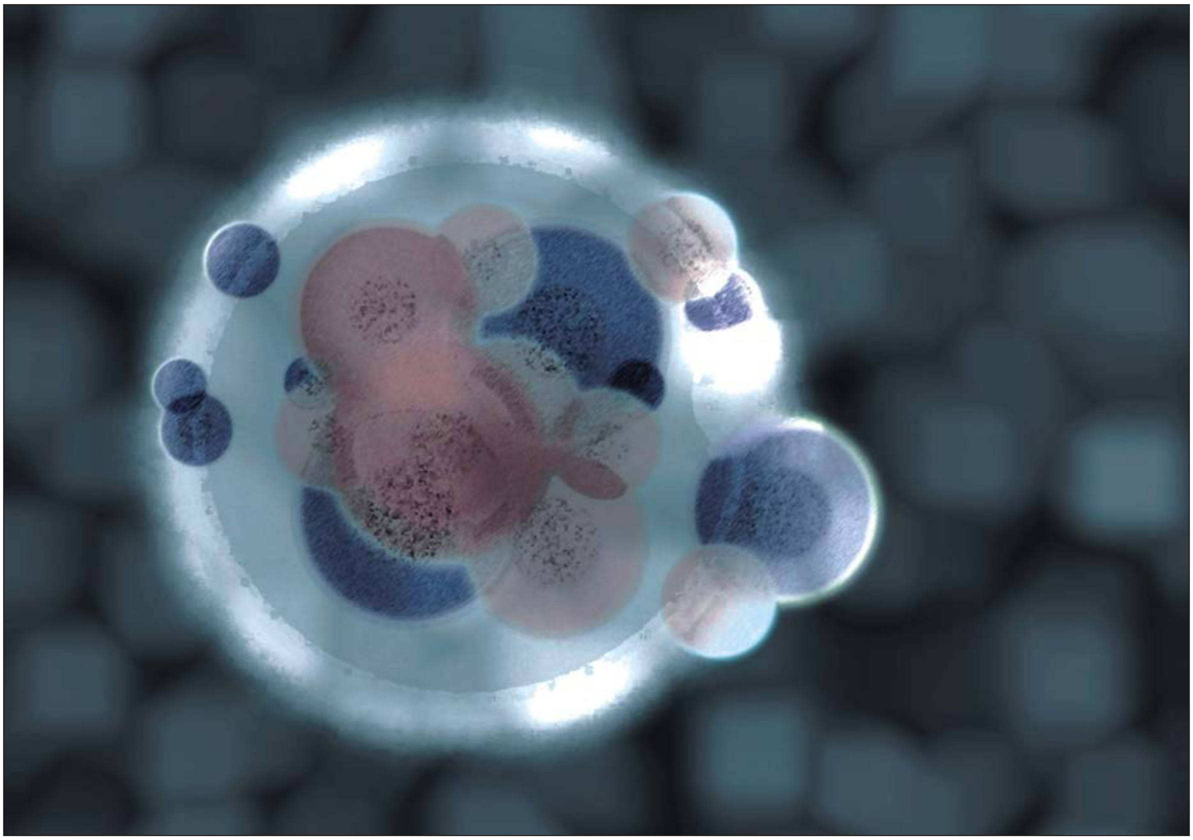

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Anatomy of *Citrus* sp. and *Kalanchoe tubiflora* Tumor Tissues

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Abstract: Plants of the orange Piralima *Citrus sinensis*, *Osbeck* develop symptoms of an anomaly in certain branches that distinguish them from the rest of the plant. Among the various hypotheses raised to explain the cause of this disease is that of *Agrobacterium tumefaciens*, carrier of the Ti plasmid. Examinations were performed under an optical microscope of the tumors tissue structure that occurs in citrus branches compared with the tumors caused by the Ti plasmid in *Kalanchoe tubiflora*. The tumors produced in *K. tubiflora*, caused by different isolates of *A. tumefaciens* containing the Ti plasmid, revealed the same cell disorganization between the epidermis and the cambial region as the tumors produced in citrus. In the region closer to the cambium, in addition to the disorganization it was observed that the tracheal elements presented greater affinity to safranine. Both in *K. tubiflora* and in Piralima plants, the increase in the tumor volume resulted in hyperplasia caused by the abnormal activity of the vascular cambium. Anatomic destruction of the peripheral tissues of the organs occurred in both plants.

Key words: *Citrus* sp. tumor tissue structure, Ti plasmid, *Kalanchoe tubiflora*, anatomy

INTRODUCTION

More than 70% of citrus production in Brazil is used by the juice industry. The internal market consumes about 28% of the production. Piralima orange plants, from a batch of 64 mother plants, planted in the experimental field of the Coordination of Integral Technical Assistance (CATI-Tietê/SP), occasionally develop symptoms of an anomaly in certain branches that distinguish them from the rest of the plant. These branches become more elongated than the normal ones acquiring an s or circular shape, resembling a whip (Costa *et al*., 1982). Evidence obtained previously indicated that the whip is not an infectious disease or if there is a pathogen associated with it, it is difficult to transmit by tissue union, although there is invariably perpetuation of the anomalous condition by the gall. Galls from anomalous Piralima branches develop broom appearances that when necrosed frequently die. Studies by Costa *et al*. (1982) showed that the whip might become a more serious problem in the future. Currently the symptoms have already been found both in Piralima and in Pêra oranges that are very important economically (Costa *et al*., 1998).

The various hypotheses raised to explain the cause of this disease include one referring to *A. tumefaciens*, carrier of the Ti plasmid (Pacheco and Silva, 1994). *A. tumefaciens* is the causal agent of crown gall disease that affects most dicotyledonous plants and is characterized by growth of tumors in the region between the plant stem and root (crown) (Schrammeijer *et al*., 2003). The development of tumors is the result of a natural transfer process of *Agrobacterium* sp. genes to the genome of the host plant (Winans, 1992; Sheng and Citovsky, 1996; Stafford, 2000). Two Ti plasmid regions are directly involved in tumor induction. The T-region, which corresponds to the segment of transferred DNA to the plant cells and the virulence (*vir*) region, contain genes involved in the synthesis of proteins required for T-region transfer. This region, when transferred and integrated to the plant cell genome, is called T-DNA (transferred DNA). The genes present in T-DNA encode enzymes involved in the biosynthesis of plant growth regulators, auxin and cytokinin. The synthesis of these regulators by transformed cells results in a hormonal imbalance, leading to the development of tumors where the infection occurred (Tzfira and Citovsky, 2000; Zupan *et al*., 2000). Another group of genes presented in T-DNA encodes enzymes required for opine synthesis, which are specifically catabolised by the colonizing bacterium as the source of nutrients (Binns *et al*., 1992).

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Initially, researchers associated the development of crown gall to animal cancer, which stimulated numerous researches trying to elucidate its induction mechanisms and formation. Those studies concluded that the formation of crown gall is the result of a natural process of genes transfer from the bacteria to the vegetable cell, that start to synthesize proteins stimulating the cellular division in the infection branch. The knowledge ever since generated culminated with a quite deepened understanding of that parasitism, considered now a system-model for studies of the interactions pathogen-host in plant (Andrade *et al*., 2003).

Previous studies showed the evidence of agrobacterial infection within the marker plants using molecular and biochemical characterization of opines and the presence of DNA sequences specific for T-DNA and *vir* genes (Pacheco *et al*., 2006).

The study described here examined the anatomy of two plants infected by *A. tumefaciens* containing Ti plasmids causing tumor tissue. The cross-section at the base and the middle of the tumors and its correlation with the morphology of cells was investigated.

MATERIALS AND METHODS

Plant material: One-year-old Piralima orange [*Citrus sinensis* (L.) Osbeck], presenting a tumor on the graft and in the branches were provided by the Virology Laboratory at the Campinas Agronomic Institute, (Campinas-SP) and *K. tubiflora* plants were inoculated with *A. tumefaciens* 711 used to infect plants, which was isolated from *Chrysantemum morifolium* plants.

Optical microscope: In order to observe the structural modifications in the tumors in Piralima citrus cultivar and *K. tubiflora*, stained sections were carried out to study under optical microscope. The regions of the healthy internode of the two plant species studied were used as positive controls. The materials collected were fixed in 50° ethylic formaldehyde (Saas, 1951). The thickness of sections ranged from 10 to 15 μm. The slides were deparafinated with xylol, the material was stained with safranine (lignin) and fast-green (cellulose + cytoplasm) and finally placed in synthetic resin. Sections of fresh material were made by hand and by a sliding microtome to compare with fixed and dehydrated material. These sections were stained with green iodine and Congo Red according to Dop and Gautie (1928).

All of the tumor sections, from both Piralima orange and *K. tubiflora* were examined microscopically for their anatomy.

RESULTS

Tumor tissues of Piralima orange [*Citrus sinensis* (L.) Osbeck] (Fig. 1) and *K. tubiflora* (Fig. 2) infected with *Agrobacterium* were examined for correlation of their morphology and anatomy. The analysis of the anatomic structure in the region of the healthy internode of the *K. tubiflora* species showed that the epidermis is uniseried and the collenchyma has 1 to 3 layers of cells and the cortical parenchyma has about 9 layers of cells (Fig. 3A). The vascular tissues were arranged in bundles of the open collateral type and the medulla was verified internally. Fig. 3B shows the detail of the installation of the vascular cambium from the procambium (fasciculated cambium) and of the interfasciculated parenchyma.

Comparison of Fig. 3B with Fig. 4B shows that the alterations induced by the *A. tumefaciens* 711 are reflected in the disorganization of the cortical collenchyma epidermis and vascular tissues. The activity in the

Fig. 1: Tumors in the Piralima (*Citrus sinensis*, (L.) Osbeck) branches

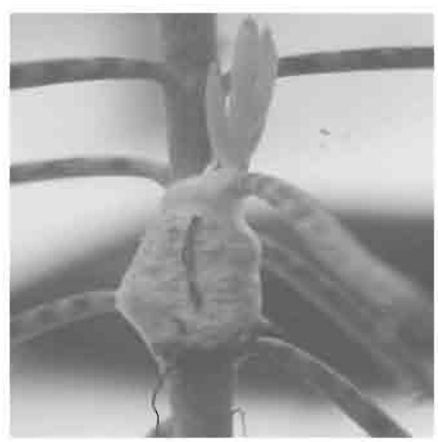

Fig. 2: Tumors in the branches of *K. tubiflora* introduced by the *A. tumefaciens* 711 isolate

A **B**

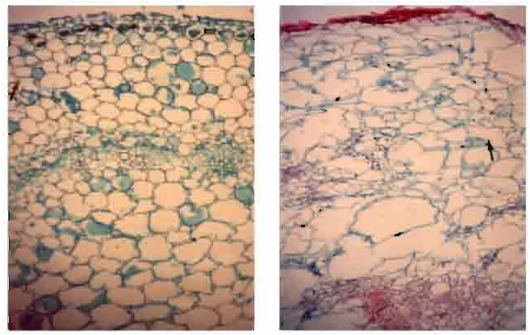

Fig. 3: Transversal sections of the internode of the *K. tubiflora* species (A). Anatomic structure of healthy internode compared to the structure altered by the tumor induced *A. tumefaciens* (B). Observe the anatomic destruction of the peripheral region of the vascular system (arrow)

A B

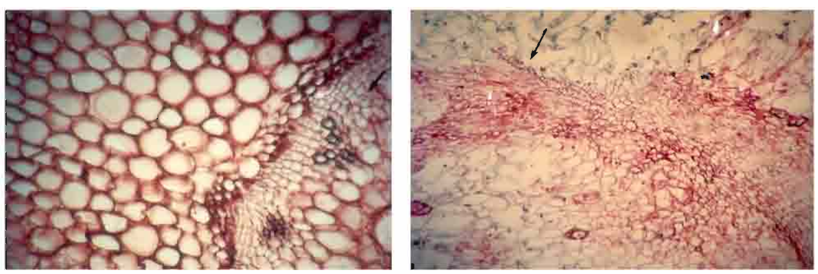

Fig. 4: Transversal sections of the healthy *K. tubiflora* internode, showing the organized vascular cylinder (A). Transversal cut of the *K. tubiflora* internode with tumor induced by *Agrobacterium tumefaciens*, showing the disorganization of the vascular cylinder and the presence of tracheal elements with irregular lignin deposits (arrow), resulting from the abnormal activity of the cambium tissue (B)

A B

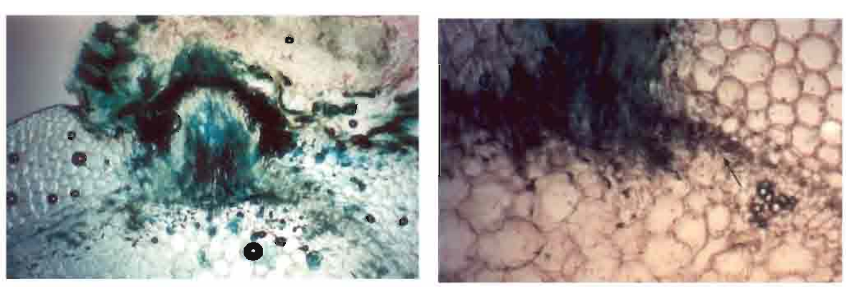

Fig. 5: Transversal sections in the tumor region of the *K. tubiflora* species inoculated by the *Agrobacterium tumefaciens* 711 isolate. Observe induction of root formation of endogenous origin close to the cambium region (B) (arrow)

vascular cambium was altered, because the tracheal elements formed presented irregularly deposited lignin (Fig. 4B). Transversal sections cut of the healthy *K. tubiflora* internode, show the organized vascular cylinder (Fig. 4A).

The presence of parenchyma cells whose contents showed affinity to safranine was also observed suggesting a possible accumulation of phenolic compounds. The transversal sections cut in the region of the tumors in the *K. tubiflora* species showed

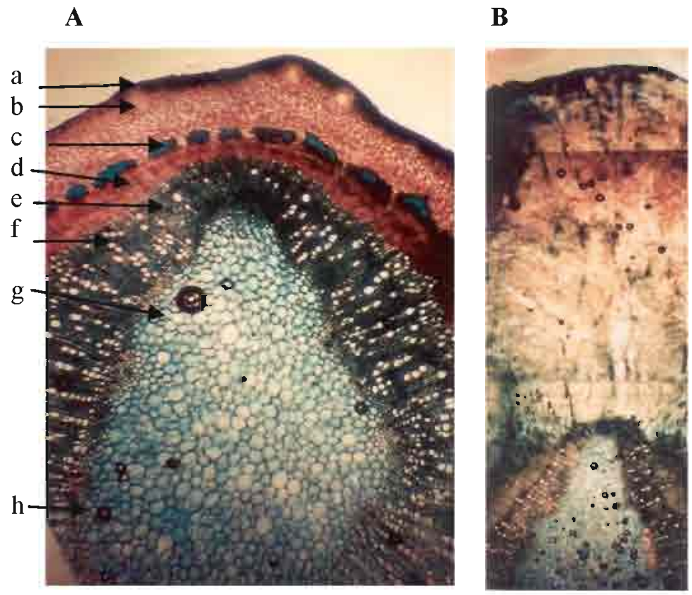

Fig. 6: (A) Transversal sections of the healthy node region of Piralima, showing the uniseried epidermis (a); collenchyma (b); cortical parenchyma (c); groups of lignified perivascular fibers (d); phloem (e); cambium band (f); xylem (g) and medulla (h). (B) Transversal cut of a region is similar to the previous figure, but with the formation of a tumor. Observe that the whole peripheral region shown in A (a, b, c, d) was eliminated and the secondary xylem well developed with large proliferation of parenchyma cells and some tracheal elements

that root formation of endogenous origin was induced from the cambial region (Fig. 5A and B).

Transversal cut sections in the healthy node region of Piralima showed that the epidermis is unisseriate, the collenchyma presented about six layers of cells, where the oil cavity occurred and the cortical parenchyma presented approximately 13 cell layers. The presence of groups of lignified perivascular fibers forming a continuous cylinder was observed and then the phloem, cambium box, xylem and finally the medulla (Fig. 6A).

The transversal cut sections through the nodal region altered by the tumors in the Piralima species, supposedly induced by Ti plasmid, showed that there were drastic alterations in the anatomic structure, apparently induced by the abnormal activity of the vascular cambium that came to form the secondary xylem that was well developed due to the great proliferation of parenchyma cells. This proliferation led to the elimination of the entire peripheral region previously consisting of the epidermis, chlorenchyma, cortical parenchyma, perivascular fibers, that was substituted by the peridermis (Fig. 6B).

DISCUSSION

The occurrence of tumors in citrus is extremely interesting and reveals several characteristics that should be discussed. It is an anomalous process, but is potentially serious for citrus culture.

The anatomy of the citrus teratoma whose pathogen has not yet been proved, when contrasted with the tumor anatomy of *K. tubiflora*, induced by the Ti plasmid, showed that the tumors produced in *K. tubiflora* presented the same disorganization of the cell region between the epidermis and the cambial region. The tracheal elements in the region close to the cambium presented greater affinity with safranine in addition to the disorganization. The increase in tumor volume in both *K. tubiflora* and Piralima resulted in hyperplasia caused by the abnormal activity of the vascular cambium. There was anatomic destruction of the peripheral tissues of the organs in both plants.

The accumulation of products with great affinity for safranine, probably phenylpropanoids or derivates, seems to show decided intervention of a pathogenic entity in the life of the shichemical acid and its variations. The reaction with the formation of acetosyringone and others would contribute to successful tumor formation (Pacheco and Silva, 1994).

Phenolic compounds (ketosyringone and hydroxi-ketosyringone) are the most important inducers of virulence in agrobacteria. The acidic pH (5.2 to 5.8), sugars (such as glucose and galactose) and amino acids were also characterized as activators of *vir* region

(Winans, 1992), but are not capable to stimulate the induction in the absence of phenolic compounds. These chemical inducers are characteristic of harmed tissues: phenolic compounds and sugars are precursors of important components of the cellular wall such as lignin and cellulose and as the vacuoles occupy most of the cellular volume and are usually acids, the plant sap tends to be acid (Sheng and Citovsky, 1996; Winans, 1992). The process of tumor formation in the citrus branches presented a series of structural similarities with *K. tubiflora* tumor that is caused by Ti plasmid, including hyperplasia and hypertrophy of the cells situated between the cambium and the epidermis, the accumulation of compounds with great affinity for the safranine stain, thickening of the cortical region and cellular disorder.

The morphology of a tumor induced by agrobacteria is highly controlled by the level of cytokinins and auxins in the expression of the oncogenes within the transformed cells (Binns *et al*., 1992). The teratomas contain a cytokinin/auxin hormonal balance favorable to gall formation, while the undifferentiated tumors produce a level of auxin that inhibits the development of these galls (Amasino and Miller, 1982; Liu and Nester, 2006). It is important to remember that the morphology of a tumor depends exclusively on a hormonal balance of the plants at the level of the site of infection (wound location). The cytokinins are produced inside the roots and transported through the plant (stem and leaves) by the xylem. The auxins are produced mainly in apical zones of the stem and young leaves and transported to the plant base (stem and roots) to a cell-to-cell polar system (Hooykaas and Beijersbergen, 1994). It is conceivable that because of the polar transport of these two hormones, the tumor morphology is different depending on the infection location in the plant that depends on the plant species and on the *A. tumefaciens*. Auxin and cytokinin production by the integration of the T-DNA region combined with the endogenous hormone level and the location of the inoculation in the plant may determine the morphological organization of the tumors (Schröder *et al*., 1984). Thus in *Agrobacterium*, the tumor morphology is essentially determined by the T-DNA and the endogenous hormone levels in the infested plant.

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