Safargali (Stubborn) Disease and an Associated Tumor-Inducing Agent in the U.A.R.

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FOR MANY YEARS, sweet orange [*Citrus sinensis* (L.) Osb.] trees affected with safargali disease were thought to be a declining and otherwise unsatisfactory variety. They were named safargali variety accordingly. Propagation from such trees was prohibited by law. Childs *et al.* (4) believed this disorder was caused by stubborn virus disease. Symptoms appearing on safargali trees coincide with those induced by stubborn and related diseases in several citrus-producing regions (1, 3, 5, 9).

Prevalence and Symptomatology

Safargali disease (8) is the most important disease problem of the citrus budwood certification program in the U.A.R. After several thousand citrus trees were certified free of psorosis, xyloporosis, cachexia, exocortis, gummy bark of sweet orange, and tristeza, symptoms of safargali disease began to appear in prospective virus-free mother trees. Examination of certified balady, Valencia, and navel sweet orange trees during the six years following propagation showed that 29 (18 per cent), 39 (14 per cent), and 3 (3 per cent), respectively, exhibited symptoms of safargali disease. Infected trees were grafted on the following rootstocks:

sweet orange, sweet lime (*C. limettioides* Tanaka), balady lime [*C. aurantifolia* (Christm.) Swing.], Rough lemon (*C. jambhiri* Lush.), Rangpur lime (*C. reticulata* var. *austera* hyb.), calamondin (*C. mitis* Blanco), sour orange (*C. aurantium* L.), Orlando tangelo (*C. reticulata* Blanco x *C. paradisi* Macf.), balady mandarin (*C. reticulata* Blanco), grapefruit (*C. paradisi* Macf.), trifoliate orange [*Poncirus trifoliata* (L). Raf.], and several varieties of citrange (*P. trifoliata* x *C. sinensis*). Observations of these trees indicate that safargali disease spreads under natural conditions, and that the disease can infect sweet orange trees grafted on any of these rootstocks.

Safargali-diseased trees usually develop thin chlorotic foliage as a result of premature leaf drop and poor growth. Some limbs grow horizontally, producing stunted upright twigs with short internodes, and bear small, stiff, rolled leaves. Many diseased trees have an upright habit of growth, but others are bushy and flat-topped. Leaves of diseased sweet orange trees are usually small, mottled, chlorotic, thick, and rolled upward along the midrib or perpendicular to it.

The most reliable symptom of safargali disease is the production of small, lopsided fruits of poor quality that contain a high percentage of small, brown, aborted seeds. Juice sacks are abnormally soft, smooth, and yellow-colored, and appear fermented. The peel thickness is extremely irregular, and by mid-season most fruits develop creasing of the rind.

A newly reported (13) symptom associated with the disease is the development of abnormal flowers. Under normal conditions, sweet orange trees blossom for approximately six weeks. On the other hand, safargali-diseased trees continue to bloom for more than eight weeks and 63 to 83 per cent of the flowers produced during the last four weeks have imperfect or rudimentary ovaries. In many apparently healthy trees, as well as those suspected of safargali infection, 23 to 29 per cent of the flowers had rudimentary ovaries.

Sweet orange flowers with rudimentary ovaries can be detected without being opened. The petals of normal flowers separate evenly as they start to open, whereas the petals of imperfect flowers are usually twisted inward and commence separation midway between the tip and the base (Fig. 1,A).

Autumn leaf drop is another characteristic associated with safargali disease. Late in October or during November, mesophyll collapse occurs in the leaves of navel, balady, and Valencia orange trees propagated from old or new lines. It appears first as irregular, water-soaked, light-

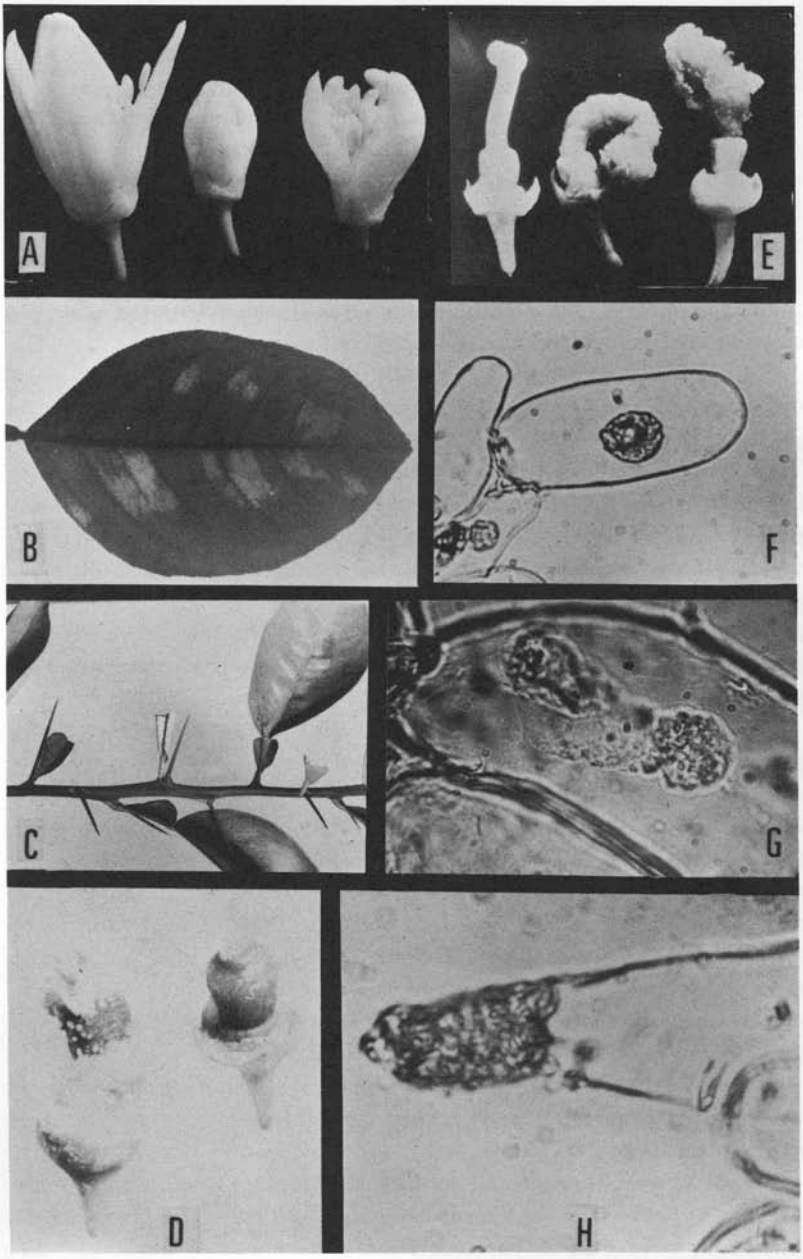
colored blotches in the leaf lamina, particularly on leaves formed during the summer or early autumn months (Fig. 1,B). Affected leaves become chlorotic and drop, leaving the petiole attached to the twig (Fig. 1,C). Later, the petiole falls, and in most cases a drop of gum-like material oozes from the leaf trace. At the same time, a severe dieback condition starts from the tip, progresses to the base of the twig, and occasionally extends to the limb. The pruning off of dead twigs during winter usually leaves affected trees with severely shortened limbs. Intense vegetative growth starts the following spring, and the trees regain their vegetative shape although flowers are sparse on such trees and, consequently, fruit production suffers. This condition has led to the abandonment of several sweet orange varieties including Youssef Soliman.

Safargali disease spreads naturally in orchards of seedling sweet orange trees. The trees grow vigorously for three to four years after planting, then chlorotic and small leaves begin to appear on a few trees. These leaves usually wilt and dry out, but generally do not abscise during autumn and winter months. Severe dieback of twigs follows, accompanied by different degrees of twig gumming. The following spring, a vigorous flush of foliage fills the gap left by the dieback of twigs. However, as the leaves mature, interveinal chlorosis develops, and part of this flush may die back late in the spring. Within a few years, tree size becomes static; in fact, affected trees often decrease in size due to severe pruning of dead twigs and branches.

Progress of the disease is quite rapid. In the first two years of fruit production, seedling trees usually produce fair crops, but as leaf mottling and dieback increase, yields drop sharply; the fruits are small, severely lopsided, and unevenly colored. As the disease intensifies, flower production decreases, fruit set is reduced by severe flower and fruit drop, and only a few fruits are left on the trees. These fruits become pale yellow as they reach maturity. The peel is abnormally thick, 1 to 4 cm, which leaves little space for carpels, and the surface is coarse and rough. Juice sacks are very dry and a light green yellow or white color. Seeds are absent in such fruits or, if present, are aborted. After a few years, affected trees become only skeletons of main limbs and branches.

Safargali disease is also considered responsible for the decline of seedling balady lime trees. The symptoms resemble those on sweet orange seedling trees described above.

Decline of sweet orange and seedling balady lime trees is much more frequent on open soils than on heavy clay. Ten years after planting on sandy soil, more than 50 per cent of the trees may be affected and un-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productive. Safargali disease has been observed in many recently planted orchards in both upper and lower Egypt.

Materials, Methods, and Results

TRANSMISSION OF SAFARGALI DISEASE.—In March, 1965, 15 one-year-old certified Valencia sweet orange trees on sour orange rootstock were inoculated with buds from each of 3 naturally safargali-infected sweet orange trees, a total of 45 trees. Twenty-four trees were left uninoculated as checks.

During the following autumn and winter months, leaf drop occurred on all 45 inoculated trees. Small mottled and chlorotic leaves appeared on 20 of the inoculated trees during the spring and summer of 1966, but the small leaf condition did not develop in the first year following inoculation. The easy transmission suggests that the causal agent of the autumn leaf-drop condition is not the same as that responsible for the small leaf condition, although they are associated.

FORMATION OF TUMORS ON DISEASED FLOWERS, FRUITS, AND TWIGS.—Both the healthy and safargali-diseased citrus trees used in this study were propagated from nucellar seedling trees, indexed and found free of psorosis, xyloporosis, cachexia, exocortis, gummy bark of sweet orange (phloem discoloration), and tristeza virus diseases. Some of these progeny trees later exhibited all the characteristic symptoms of safargali disease.

OCCURRENCE OF TUMORS.—Flowers from diseased balady, navel sweet orange, and Mexican lime trees were cultured on potato-dextrose agar. After one week, 55 to 88 per cent of the cultured flowers had developed tumors (13), mostly between the button and the calyx, or from the but-

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FIGURE 1. *A. Left: Normal flower with normal ovary and normal separation of the petals. Right: Flowers with rudimentary ovaries. Note twisting of the petals before and after flower opens. B. Sweet orange leaf showing mesophyll collapse. C. Petioles attached to the twig after autumn leaf drop of sweet orange. D. Left: Ovary and pedicel from a safargali-diseased tree cultured on PD agar for 15 days. Note tumorous growth from the pedicel and at the style scar. Right: Healthy ovary. E. Ovaries of Washington navel orange cultured on agar for 11 days. Left: Neither ovary nor pedicel developed tumors. Center: Style and stigma enlarged and covered with thin tumorous tissue, but no change in the ovary. Right: Tumor developing on style and stigma, but not extending to ovary; no tumor developed on pedicel. F. A spherical inclusion body in hypertrophied cell. Note intercellular strand of proteinaceous nature always associated with tumorous tissue. G. A plasmodium-like structure in a hypertrophied cell. H. Intercellular structure developing in tip of hypertrophied cell.*

ton of the pedicel, which caused the ovary to separate from the disc and rise on top of the tumor (Fig. 1,D). Tumors sometimes developed from the scar left by abscission of the style and from the lateral walls of the ovary. At times, tumor growths appeared on petals after flowers were cultured one to several weeks.

Tumors also formed on the styles. Approximately 10 per cent of the flowers collected from progeny trees of a Washington navel sweet orange tree, introduced from Riverside, California, under No. 6/15, and cultured on potato dextrose agar, developed tumors on the style, but not on the pedicel (Fig. 1,E). This type of tumor was not found on cultured flowers of the other 17 trees studied nor on flowers of a local nucellar navel tree. From 1 to 4 per cent of the flowers from apparently healthy sweet orange trees developed tumors when cultured, whereas from 39 to 68 per cent of the flowers from trees suspected of safargali infection developed tumors.

DEVELOPMENT OF TUMORS.—Tumor growth usually starts as a soft mass of cells of light yellow color. After a few days the tumorous tissues become light brown or tan colored, and in about 10 days enlargement stops. Some tumors have a chalky appearance, suggesting a different type of growth.

When pieces of actively growing twigs from diseased trees were cultured for 1 to 2 weeks, tumor callus grew mainly from the leaf scars. When a petiole was left attached to the piece of twig, callus tissue grew from the petiole midrib. Most twig pieces became tan colored in culture and as tumor formation started, the tissues softened. Other twig pieces became discolored, but developed no callus from the leaf scars. A few twig pieces remained green and developed whitish compact callus tissue at the cut ends, but not from the leaf scars. This is considered normal, healthy callus development in contrast to the tumorous growths described previously. Tumors failed to form on pieces of twig more than a month old when collected. Apparently the causal agent stimulated tumor formation in meristematic tissues only.

When whole fruits of safargali-diseased Mexican lime trees were cultured, a thin tumorlike growth developed on the peel of over 20 per cent of the cultured fruits. When such fruits were cut, gum pockets in the albedo were revealed.

Microscopic examination of unfixed and unstained pieces of tumor showed the tissue to be composed of thin-walled hypertrophied cells. The mean length of 212 cells was 160 μ and cell length ranged from 99 to 187 μ. Most cells contained light brown inclusion bodies of varied

shapes. In young cultures, many cells contained spherical inclusion bodies with granular structure, one to each cell (Fig. 1,F). Three-week-old tumors consisted mostly of hypertrophied cells containing elongated granular plasmodium-like structures (Fig. 1,G). On the other hand, tumors that developed from leaf scars of cultured twigs consisted mostly of small, spherical hyperplastic cells and few hypertrophied cells. These hyperplastic cells contained similar inclusion bodies.

Microscopic observation, for several days or weeks, of a hanging drop containing hypertrophied cells, revealed the formation of motile bodies within the plasmodium-like structures. These bodies remained motile for several weeks. Rupture of the spherical inclusion bodies or formation of the plasmodium-like structures was not observed.

Examination of lightly loosened hypertrophied cells revealed a network of light brown strands between the cells and a network enveloping parts of each cell (Fig. 1,H). Pressure applied to the cover slip broke the strands into very small yellow structures. When tumorous tissue was macerated in a few drops of 0.25 per cent ninhydrin solution on a glass slide and heated on steam for about 5 min, the intercellular strands stained rose color a reaction indicative of proteins. The spherical inclusion bodies and intercellular strands also gave a positive reaction with ninhydrin. On the other hand, no change in the color of the plasmodium-like structures was observed in the ninhydrin test.

Discussion

In many respects, safargali disease symptoms resemble the symptoms reported for stubborn (5, 6) and greening diseases (9). The wide divergence of symptoms exhibited by safargali-infected trees suggests that the disease has more than one form. For example, the infected progeny of one citrus clone can exhibit different symptoms. Some have an upright habit of growth, and others have flat-topped growth. Some have very small stiff leaves, resembling those of mandarin, and others have small to medium-sized leaves.

Stubborn disease in California and Morocco is characterized by the frequent appearance of acorn-shaped fruits (3, 6), a symptom seldom seen on trees infected with the greening disease in South Africa (9) or with safargali-diseased trees in the U.A.R. The existence of different strains of the causal agent may account for the different symptoms exhibited by those three diseases. It is also possible that flowers destined to give acorn-shaped fruits drop before setting fruit or soon after, and before the fruits acquire the acorn shape.

The site of tumor development on flowers of Washington navel from California differed in some instances from the sites on flowers of local trees and of Valencia trees imported as budwood from California. Some flowers of the imported Washington navel tree developed tumors on the styles, a site never observed with flowers of the other trees, including a local clone of navel orange. Because of this unusual site of tumor development, the tumor-inducing agent is considered to have more than one form or strain.

Stubborn disease is considered a virus disease on the basis of bud-transmission studies (5). Safargali disease resembles stubborn disease in being bud transmissable and in having no visible parasites in diseased tissues. However, discovery of an obligate parasite that stimulates hypertrophy and hyperplasia in cultured safargali-diseased material provides another aspect for study in the etiology of this disease.

Hypertrophied cells are free from many of the cell components that usually hamper observation of the causal agent in diseased tissues. Plasmodium-like structures as well as spherical inclusion bodies are clearly visible in hypertrophied cells without fixing or staining. Consequently, such structures can be observed microscopically over a considerable period, and changes taking place in them can be detected. To date, the only change observed in the plasmodium-like structures is the formation of organelles that remain motile for several days or weeks. This is the main reason for considering the irregular granular structures present in hypertrophied cells as plasmodium-like. These intracellular structures appear to be of chytrid nature.

Spherical bodies are found in many cells of recently developed tumors, but they decrease in number as the tumor becomes older. However, cells containing plasmodium-like structures increase in number as the tumors grow older. Both structures have never been observed in one cell. These facts suggest that the spherical bodies turn into plasmodium-like structures. To date, the change from spherical body to plasmodium-like structure has not been observed, possibly because only detached tumorous cells were observed. It was not possible to place intact hypertrophied cells under microscopical observation while the tumor was developing and enlarging.

Little is known about the chytrid-like organism associated with safargali disease, nevertheless, it, and not a virus, may be the causal agent of this disease. Additional evidence against the virus hypothesis is the presence of proteinaceous strands between hypertrophied cells. These strands can be detected very early in the development of the tumor.

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