*Review Article*

***Agrobacterium: An important bacterium in genetic engineering for gene transformation***

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ABSTRACT

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| *Agrobacterium* is not only an important bacterium in genetic engineering for gene transformation but also an important plant pathogenic bacterium. Economically significant diseases caused by *Agrobacterium* include crown galls in apples and pears, caused by *A. tumefaciens.* Agrobacteria are gram-negative, rod-shaped bacteria belonging to the class Alphaproteobacteria and the family Rhizobiaceae.These *Agrobacterium* strainscan be isolated from galls grown on MacConkey agar Carrot-disk and potato-disk bioassays can be used for pathogenicity tests.Other major crops, including crown gall disease caused by *Agrobacterium*, include many woody plants, primarily stone fruits, pome fruits, willows, and grapes. In this chapter, we critically review aspects of *Agrobacterium* such as its taxonomy, geographical distribution, transmission, host range, symptomatology, pathogenicity, biology, epidemiology, survival, and economic importance. Important components of crown gall disease management, such as cultural, mechanical, chemical,biological, and resistant/tolerant varieties also form the text of the present review. |

*Keywords: (Agrobacterium, crown gall, tumour, apple, grape)*

1. INTRODUCTION

*Agrobacterium* is an important bacterium in genetic engineering for gene transformation, but it is also an important plant pathogenic bacterium. *Agrobacterium tumefaciens* (Smith and Townsend, 1907) is mainly associated with crown galls of plants. This bacterium infects apples, roses, raspberries, peaches, etc., and is distributed worldwide. This bacterium is a gram-negative, rod-shaped, non-spore-forming, motile, aerobic bacterium that causes abnormal cell proliferation (hyperplasia), which leads to tumor formation. *Agrobacterium* is closely related to *Rhizobium* which forms nitrogen-fixing nodules on the clover and other leguminous plants. Some scientists are considering transferring *Agrobacterium* to *Rhizobium* (Hert and Jones, 2003). More than 100 different families of dicotyledonous plants are infected by the gram-negative bacterium *Agrobacterium* *tumefaciens* (Lacroix and Citovsky, 2016).

**2. TAXONOMY**

Agrobacteria are gram-negative, rod-shaped bacteria belonging to the class Alphaproteobacteria and family Rhizobiaceae, whose members represent a wide diversity of plant) pathogens and non-pathogens, including beneficial bacteria (Bosmans *et al.*,2017). The genus *Agrobacterium* was named by Conn and classified under the family Rhizobiaceae along with the genus *Rhizobium*. According to the 8th edition of Bergey’s Manual, *A. radiobacter* (non-pathogen), *A. rhizogenes*(causes hairy roots) and *A. tumefaciens* and *A. rubi*(associated with plant galls). The criteria for classifying these species are based on phenotypic characteristics and symptomatology, and new species of the genus *Agrobacterium* have been described based on rrs (i.e., the 16S rRNA gene) polyphyly and several housekeeping genes (Young *et al*.*,* 2001). *Agrobacterium arsenijevicii* and *Agrobacterium rosae* (plant galls), *Agrobacterium deltaense*, *Agrobacterium salinitolerans*(legume nodules), and *Agrobacterium bohemicum*(plant waste)were isolated from different plants*.* According to Rule 56a of the International Code of Nomenclature of Bacteria, only the Judicial Commission can place a name on the list of rejected names.In 2014 this commission had taken priority to the “the combination *Agrobacterium radiobacter* (Beijerinck and van Delden, 1902) Conn 1942 when the two species(*A.tumefaciens* and *A.radiobacter*) are to be treated as members of the same species. The type species of the genus is *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942, even if it is treated as a later heterotypic synonym of *Agrobacterium radiobacter* (Beijerinck and van Delden 1902) Conn 1942. *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942 is typified by the strain defined on the Approved Lists of Bacterial Names and by strains known to be derived from the nomenclatural type”. The type strains of *A. tumefaciens* and *A. radiobacter* represent two subspecies of the same species. In any case, this proposal does not modify the resolution of the Judicial Commission, given that the specific name *Agrobacterium tumefaciens* cannot be rejected as long as the Commission has not made such a decision. The species *A. albertimagni* is more closely related to some *Rhizobium* species, forming a rrs gene cluster that belongs to neither *Agrobacterium* nor *Rhizobium,* but to a separate genus. The reclassification of *A. albertimagni* into a new genus is yet to be studied. The remaining species currently included within the genus *Agrobacterium* formed a rrs cluster phylogenetically divergent from the genera *Allorhizobium* and *Rhizobium* also contain plant pathogenic species. Most *Agrobacterium* species include tumor-inducing species, species,non-pathogenic species, *A.salinitolerans* nodules in legume *Sesbania cannabina* and even the human pathogen *A. pusense* (Flores *et al.,*2020).

*Agrobacterium tumefaciens* (biovar 1, *Rhizobium radiobacter*), *Agrobacterium rhizogens* (biovar 2, *Rhizobium rhizogens*), and *Agrobacterium vitis* (biovar 3, *Rhizobium vitis*) are the three main pathogens of *Agrobacterium* species. *Agrobacterium rubi* (*Rhizobium rubi*) and *Agrobacterium larrymoorei* (*Rhizobium larrymoorei*) are minor pathogen species that reside in the soil. The crown gall of grapes, mainly caused by *Rhizobium vitis* (Ti) Agrobacterium tumefaciens biovar 3 (Smith and Townsend 1907) Conn 1942], is the most important bacterial disease of grapevines worldwide. However, *A. tumefaciens* (the predominant causal agent of crown galls of other crops) has also been isolated from galls on grapes and is associated with the disease at a much lower frequency than *A. vitis* (Vizitiu *et al.,*2012).

Bosmans *et al.* (2017) studied three large clusters in the genus *Agrobacterium* (Table 1) corresponding to biovars 1, 2, and 3. Biovars are determined by chromosomal genes and not plasmids, and therefore, better reflect phylogenetic relationships. As a result, tumorigenic, rhizogenic, and non-pathogenic strains can be found within the same biovar. More specifically, biovar 1 contained strains of *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter*, including the type strains of *A. tumefaciens* and *A. radiobacter*. Biovar 2 also contained strains of all three species, including the type strain of *A. rhizogenes*. Biovar 3 contained *A. tumefaciens* and *A. vitis* strains.

**Table 1: : Classification of Agrobacterium strains [modified from Ophel and Yerr(1990); Lindstrom *et al.*(2007);Portier *et al.*(2006);Slater *et al*.(2009) ;Lindstrom and Young(2011) and Bosmans *et al.*(2017)]**

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| **Old classification** | **Biovar classification** | **New classification** |
| *Agrobacterium* *tumefaciens*  *Agrobacterium* *radiobacter* | Biovar 1: tumorigenic (*A. tumefaciens* C58), rhizogenic (*A. rhizogenes*), and avirulent (*A. radiobacter*) strains, includes type strains of *A. tumefaciens* and *A. radiobacter* (Genomovar G4) | *Agrobacterium tumefaciens* species complex |
| *Agrobacterium* *rhizogenes* | Biovar 2: tumorigenic (*A. tumefaciens*), rhizogenic (*A. rhizogenes*) and avirulent (*A. radiobacter* K84) strains, include type strain of *A. rhizogenes* | *Rhizobium* *rhizogenes* |
| *Agrobacterium* *rubi* | Biovar 3: tumorigenic on Vitis (*A. tumefaciens* and *A. vitis* S4) and these are Genomovars | *Agrobacterium* *rubi* |
| *Agrobacterium* *vitis* | *Agrobacterium* *vitis* |
| *Agrobacterium* *larrymoorei* | *Agrobacterium larrymoorei* |

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**3. GEOGRAPHICAL DISTRIBUTION**

*Agrobacterium tumefaciens* is found on all continents, but the countries listed below have the most occurrences of crown galls (Commonwealth Mycological Institute. Distribution maps of plant diseases: *Agrobacterium tumefaciens*. Online: <http://www.cabi.org/dmpd/abstract/20056500137> ).The details are as follows:

**i. Europe**

Austria, Azores, Belgium, Britain, Northern Ireland, Bulgaria, Cyprus, Czechoslovakia, Denmark, Finland, France, Germany, Greece, Hungary, Italy, Netherlands, Norway, Poland, Romania, Spain, Sweden, Switzerland, USSR (Estonia) (Ukraine)Caucasus), and Yugoslavia.

**ii. Asia**

Afghanistan, China (S.) (Honan) (N.E.), India, Indonesia, Iran, Israel, Japan, Korea, Lebanon, Malaysia (Sarawak), Saudi Arabia, Sri Lanka, Syria, Turkey, Union of Soviet Socialist Republics (Central Asia, Western Siberia).

**iii. Africa**

Algeria, Egypt, Ethiopia, Kenya, Libya, Malawi, Morocco, Mozambique, Rhodesia, Seychelles, Somalia, South Africa, Tanzania, Uganda, and Zambia.

**iv. North America**

Canada, Mexico, United States of America.

**v. Central America and Caribbean**

Bermuda, Cuba, French Antilles, Guadeloupe, Jamaica, Puerto Rico.

**vi. South America**

Argentina, Bolivia, Brazil (São Paulo, Pernambuco), Colombia, Chile, Guyana, Peru, Uruguay, and Venezuela.

**vii. Oceania**

Australia (All territories), New Zealand.

Bosmans *et al.* (2017) reported that Hairy Root Disease (HRD) is prevalent in several European countries including Austria, Belgium, Denmark, France, Greece, the Netherlands, Poland, Switzerland, and the UK. A survey performed in Flanders (Belgium) in 2014 indicated that 33% of the tomato, cucumber, and aubergine producers were confronted with HRD. In the Netherlands, nearly half of hydroponic growers had to deal with HRD. Since 2013, cucumber and tomato crops in the Russian Federation have also been affected by HRD, which has become a major problem in the greenhouse vegetable industry. In addition, hairy roots have been reported in Japan, New Zealand, and the USA.

**4. TRANSMISSION**

The crown gall bacterium overwinters in galls and plant debris in soil, and may also survive saprophytically in soil for several years. Host plant infections usually occur through lenticels or wounds made by various cultural practices, grafting, or insects. Chewing insects can also carry bacteria from plants to plants. Once infection occurs, genetic material from the bacterium transforms into host cells, which are induced to divide and enlarge in an unregulated manner. Once transformed, plant cells continue to divide. As galls weather or decay, bacteria return to the soil, thereby completing the disease cycle. Bacteria are disseminated over long distances in diseased planting stocks or in infested soils. The pathogen can be disseminated in plant debris, soil, water, plant propagation materials, equipment, storage sheds, etc. The disease can spread in nurseries or farms through practices such as budding, grafting, and planting. Infection: Infection occurs when the inoculum contacts wounds, natural (e.g., growth cracks), or caused by equipment, such as plowing or pruning roots when planting and harvesting for the market. Bacteria multiply in wounds when moisture is present and the temperature is not extreme. Bacteria attach to plant cells and transfer a portion of the Ti plasmid, the tumor-inducing gene (TDNA), to plant cells. This results in the overexpression of plant hormones that cause gall formation. Multiple galls on grapes result from a systemic infection with *Agrobacterium vitis* (Vizitiu *et al.,*2012).

**5. HOST RANGE AND SYMPTOMATOLOGY**

*Agrobacterium* causes crown galls in many woody plants, primarily stone fruits, pome fruits, willows, and grapes (*A. tumefaciens* or biovar 1), hairy roots in apples (*A. rhizogenes* or biovar 2), and cane galls in raspberries and blackberries (*A. rubi*). The type of symptoms produced is determined not by the species of *Agrobacterium* but by the kind of plasmid they carry: bacteria carrying a tumor-inducing (Ti) plasmid that induces crown gall, whereas bacteria carrying a root-inducing (Ri) plasmid induce hairy root symptoms. Thus, strains of all species can carry the Ti plasmid and can, therefore, cause crown gall, but so far, only strains of *A. tumefaciens* and *A. rhizogenes* have been found to contain the Ri plasmid and induce hairy root.*Agrobacterium. radiobacter* does not cause disease because it lacks a plasmid (Agrios,2005). Crown gall is one of several plant tumor diseases typified by non-self-limiting tissue overgrowth, usually on the roots and bottom portions of stems of woody plants (Table 2). Tumors appear rough on the surface with semi-soft, smooth, and spongy inner layers of tissue. With age, tumors are easily dislodged, and their outer layers are friable. Unlike other tumor diseases, crown galls are the result of genetic transformation caused by *Agrobacterium* (Lacroix and Citovsky,2013).

**Table 2. Species currently included in the genus *Agrobacterium* and species causing tumors or hairy roots currently included**

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| **Species** | **Source of isolation** |
| Genus *Agrobacterium* |  |
| 1. *radiobacter* | Soil and plant rhizosphere |
| 1. *tumefaciens* | Apple,Apricot and cherry (Ali *et al.,*2010 ;Abd-el Aziz *et al*.*,*2021) |
| 1. *rubi* | Raspberries and blackberries (Agrios,2005) |
| 1. *larrymoorei* | *Ficus* *benjamina* tumours |
| 1. *albertimagni* | *Potamogeton* *pectinatus* |
| 1. *fabrum* | *Prunus sp. Humulus lupulus, Euonymus alata, Rubus macropetalus* tumours |
| 1. *pusense* | *Cicer arietinum* rhizosphere |
| 1. *nepotum* | *Prunus, Vitis and Rubus* tumours |
| 1. *tumefaciens* | *Peach (Ali et al.,*2016) |
| 1. *skierniewicense* | *Chrysanthemum and Prunus tumours* |
| 1. *arsenijevicii* | *Prunus and Rubus tumours* |
| 1. *deltaense* | *Sesbania cannabina* nodules (Flores *et*.,2020) |
| 1. *salinitolerans* |
| 1. *bohemicum* | *Papaver somniferum* |
| 1. *rosae* | *Rosa x hybrida tumours* |
| *A.vitis* | Grape vine (Vizitiu *et al.,*2012) |
| *Agrobacterium* biovar 1 strains | Tomato and cucumber (Bosmans *et al.*,2017) |

**Symptoms produced by *Agrobacterium* species on different hosts**

Small, round hyperplasias or overgrowths are first observed on infected plants and may occur on roots, crowns, stems, and trunks. They usually occur close to the ground line; however, in some cases, galls may be found at considerable distances above the soil line. Depending upon the host, galls may reach diameters of 13 cm or more. Older galls are brownish and frequently exhibit decayed regions as they enlarge in size. They may be somewhat spongy in texture, with rough knobby surfaces. The effect of crown gall severity on plant health depends on a number of factors, such as when the infection occurs, the part of the host plant that was infected, and the number of infection sites. Stunting is a common symptom when young trees, such as walnuts, are heavily infected and more than 50% of the crown region is covered with galls. Later, such trees may blow over under strong winds. In addition, fungi may enter galls and infect trees, causing additional problems. If only a few galls develop on the roots, then no apparent effect on tree vigor may be observed.

Crown galls on grape vines are caused by *A.tumefaciens* and *A.vitis.* The disease occurs most frequently by the appearance of the roots and stems of some tumors, small at first, as a pea, soft, spongy, and less consistent, with a smooth, albic or yellow – green, composed of parenchymatous tissue, but they turn dark, hard, rough, and woody. These tumors can occur in all organs of herbaceous or woody plants; however, at the end of the growing season, they fall. Sometimes, tumors are persistent and become increasingly higher each year (Vizitiu *et al.,*2012).

Hydroponically grown cucumber and melon plants, as well as aubergine and tomato crops, are affected by a root disorder known as ‘hairy root disease’ (HRD; also known as ‘hairy roots, ‘ ‘crazy roots, ’ and ‘root mat’). HRD is characterized by extensive root proliferation within the rockwool cube and across the rockwool slab surface, which leads to strong vegetative growth and reduced fruit production. Occasionally, plants with HRD grow normally, and the yield is unaffected. Nevertheless, in most cases yield is severely reduced, e.g. more than 10% in tomato production. Owing to the persistent nature of HRD, an infested greenhouse is very likely to be reinfested in subsequent seasons, with increasing annual economic losses year by year (Bosmans *et al.*,2017).

In the early stages of development, the galls in roses appear as tumor-like swellings that are more or less spherical, white or greyish-colored, rough, spongy (soft), and wart-like. As galls age, they become dark brown to black, hard, rough, and woody in color. If the infection is severe, plants may be stunted or show various deficiency symptoms due to impaired uptake and transport of nutrients and water (Hert and Jones,2003).

**6. PATHOGENICITY/PATHOGENIC CYCLE/PATHOGEN BIOLOGY**

Bacteria enter the host plant through the fresh wounds. Wounds that commonly serve as infection sites are those made during cultivation, pruning, harvesting, or machinery operations, or are a result of freezing injury, growth cracks, soil insects, or any other factor that causes injury to plant tissues. The bacterium is most commonly introduced into a planting site or in a planting material. The disease was named crown gall because of the large tumor-like swellings or galls, which typically develop at the crown as a result of grafting. Galls can also form on stems and shoots, although aerial galls are uncommon. Agrobacterium tumefaciens is abundant in the outer portions of primary galls, which are often sloughed into the soil. When the sloughed tissue comes into contact with the wounded healthy tissue of a susceptible host, the bacterium enters the plant and induces gall formation, thereby completing the disease cycle. Tissue surrounding the gall may appear healthy; however, it has been shown that the bacterium can spread systemically throughout the whole plant. In the early stages of development, galls appear as tumor-like swellings that are more or less spherical, white or ash-colored, rough, spongy (soft), and wart-like. Later, it turned dark brown to black, hard, rough, and woody. Plants may be stunted or show various deficiency symptoms owing to the impaired uptake and transport of nutrients and water. The bacterium is a soil-borne pathogen that can persist in soil or plant debris for up to 3 years. The bacterium survives best in moist alkaline soils, and is especially severe in loose sandy or loam soils (Hert and Jones,2003).

*Agrobacterium tumefaciens* with its chromosomal virulence genes (chv genes) that are involved in pathogenicity in disease development, such as virulence (vir genes) and crown gall disease (T-DNA), are located on a large plasmid, termed the tumor-inducing plasmid (Ti plasmid). Acetosyringones, or wound phenolics released by the plant, activate virulence genes (chv and vir genes) as well as endonucleases that excise the T-DNA. Chv genes are responsible for the attachment of bacteria to plant cells at wound sites. Vir genes are responsible for the preparation of T-DNA and its exact genes (Hert and Jones,2003).

The crown gall bacterium overwinters in galls and plant debris in soil, and may also survive saprophytically in soil for several years. Host plant infections usually occur through lenticels or wounds made by various cultural practices, grafting, or insects. Chewing insects can also carry bacteria from plants to plants. Once infection occurs, genetic material from the bacterium transforms into host cells, which are induced to divide and enlarge in an unregulated manner. Once transformed, plant cells continue to divide. As galls weather or decay, bacteria return to the soil, thereby completing the disease cycle. Bacteria are disseminated over long distances in diseased planting stocks or in infested soils.

*Agrobacterium tumefaciens* is a gram-negative, rod-shaped bacterium that resides in soil preferably on the surface of roots, and the ability of *A. tumefaciens* to transfer its tumor-inducing genes (oncogenes) into the host plant cell culminates in the integration of the oncogenes into the plant chromosomes at one or more sites. The products of integrated oncogenes modify the synthesis of and sensitivity to the plant growth regulators cytokinin and auxin, causing abnormal proliferation of the transformed cells and resulting in neoplastic growth (Agrios,2005).

Pathogenic genes are most often located in large tumors that are induced by plasmids (pTi). During infection, part of this plasmid (T-DNA) is transferred and inserted into the core of the plant DNA. It is well known that strains of *Agrobacterium vitis* and *Agrobacterium tumefaciens* are resistant to 2% NaCl, while rhizogens *Agrobacterium* strains are not (Vizitiu 2011). Opine is a unique natural substance, pseudoamino acids such as octopine, nopaline, and agrocinopine, which serve as nutrients, carbon and nitrogen sources, and specific substances that increase the pathogenicity of the bacteria. Strains of *Agrobacterium vitis* strains may carry octopine, nopaline, or vitopine Ti plasmids. Where necessary, pathogenic strains have the ability to cause crown gall tumors or the hairy root condition, previously attributed to strains using the names “*Agrobacterium tumefaciens*” and “*Agrobacterium rhizogenes*,” which is indicated by reference to the tumorigenic or rhizogenic ability, respectively, of strains in *Agrobacterium*. Nonpathogenic strains previously named *Agrobacterium radiobacter* are referred to as nonpathogenic strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* or as nonpathogenic *Agrobacterium* if the species designation has not been identified. Ti or Ri plasmids determine the pathogenic status of the strains. Species comprising pathogenic or nonpathogenic strains can be reported as tumorigenic as a (Ti strain) or (Ti), rhizogenic as a (Ri strain) or (Ri), or as nonpathogenic strains of the species where relevant.

Sujatha (2014) studied *Agrobacterium* pathogenicity and reported that this bacterium perceives plant-derived signals to activate its virulence genes that transfer Transferred DNA (T-DNA) from its tumor-inducing (Ti) plasmid into the plant nucleus, leading to the production of indole-3-acetic acid (IAA), cytokinin (CK), and opines. These hormones stimulate plant growth and result in tumor formation. Opines serve as nutrient sources and signals to initiate quorum sensing (QS) to further promote virulence and opine metabolism. Plant-derived signals including γ-amino butyric acid and salicylic acid (SA) will also be recognized to activate quorum quenching that reduces the level of QS signals, thereby avoiding the elicitation of plant defense and preserving energy.

Under the influence of the expressed T-DNA genes and the subsequent increase in auxin and cytokinin levels, the transformed plant tissue undergoes uncontrolled cell division, resulting in the crown gall. Newly formed tissue presents remarkable features that differentiate it from the surrounding tissue. Recent studies have demonstrated the changes that occur in transformed tissues at the molecular and biochemical levels. The formation of crown gall tumors is an extreme developmental change that requires increased transport and metabolic fluxes achieved via genome-wide effects. For example, the concentrations of many anions, sugars, and amino acids are higher in tumors than in normal cells, which correlates with the changes in the expression of specific enzymes and solute transporters. Similar to many animal tumors, crown gall tumors and their interface with the surrounding tissues are characterized by strong vascularization, and vascular bundles consisting of both the phloem and xylem ensure connection between tumors and the rest of the host plant, thus enhancing water and solute transport. Crown galls become nutritional sinks that depend on nutrients and water in the plant on which they develop. Indeed, tumors produce carbon and nitrogen heterotrophically (mostly from glucose and amino acids), and gain energy largely anaerobically. Whereas plant defense reaction pathways are activated during early *Agrobacterium* infection and crown gall tumor development, no extensive necrosis is usually observed. It seems that host defense, which mostly involves salicylic acid- and ethylene-dependent pathways, is offset by hormonal changes, which are likely auxin-dependent, caused by tumor growth. Furthermore, although *Agrobacterium* infection initially elicits RNA silencing, which represents the host defense against foreign DNA, this defense response is suppressed in tumors, probably due to the high levels of auxin and cytokinin, which reprogram the transformed differentiated cells into undifferentiated dividing cell(Agrios,2005).

**7. ISOLATION *Agrobacterium***

The crown gall samples are to be rinsed with tap water to remove hazardous materials and other soil particles. A solution of 10% sodium hypochlorite (bleach) solution to be prepared. The gall samples are to be immersed in the solution for 3-5 min but, depending on the nature of the galls. Subsequently, the galls are to be washed with sterilized distilled water to remove traces of bleach solution. The crown gall samples are to be kept in sterilized distilled water for 5-6 days to soften them for sample collection. The galls are to be chopped into small pieces and kept in sterilized distilled water for 2-3 days (Ali *et al.*,2010). Crown gall extracts will be cultured on MacConkey medium (Bopp *et al.,*1999). Galls extracts are to be taken out from the sterilized water. The galls will be cut into small pieces using a sterilized blade. Small pieces of samples will be picked by a loop and inoculated into MacConkey medium using the streak plate method. Two replicates are to be produced for each sample. The Petri plate will be kept in an incubator at 28 °C for 2-3 days. Sub-culturing on MacConkey media

The initial bacterial culture is to be subcultured on MacConkey medium to produce a pure culture of Agrobacterium. For sub-culturing, a single colony is to be picked from each plate with the help of a loop and inoculated into MacConkey media using the streak plate method. The process is to be repeated continuously, and from each plate, one replicate is formed. After sub culturing, the Petri plates will be kept in an incubator for two to three days at 28 °C. The pure culture that appears on MacConkey medium will be used for different tests to confirm *Agrobacterium tumefaciens*.

**8**. **IDENTIFICATION OF *Agrobacterium tumefaciens***

The bacterial species can be identified from certain biochemical and physiological characteristics by observing their physiological and morphological characteristics, such as texture, color, and shape, of many bacterial colonies were taken into consideration. Biochemical tests, such as the potassium hydroxide test, gram staining, pathogenicity tests (carrot-disc bioassay and potato-disc bioassay), and antibiotic sensitivity tests, can be carried out for the identification of *Agrobacterium tumefaciens* (Ali *et al.,*2012). *Agrobacterium tumefaciens* can be isolated from different plant tissues, including the stem, leaf, and crown gall samples of aster, galls of apricot, galls of rose, root nodules of *Vicia faba* and tobacco. Different selection media, including MacConkey medium and yeast extract mannitol agar (YEMA), have been utilized for the isolation of *A. tumefaciens* from plant samples. Crown galls have been isolated from different plant species and belong to different dicotyledonous plants i.e. *Tectona grandis*, *Artocarpus heterophyllus*, *Anthcephalus codomba*, *Terminalis arjuna*, *Rosa chinensis* and *Solanum lycopersicum*. The characterization and identification of *Agrobacterium tumefaciens* is usually conducted using morphological, biochemical, pathogenicity, antibiotic sensitivity, and molecular methods. Morphological methods include observations based on size, shape, colony surface, color, opaqueness, elevation, consistency, and margin type. Biochemical methods for bacterial identification are also the method of choice for the identification and confirmation of bacterial samples. Biochemical tests are usually conducted according to the Bergey’s Manual of Determinative Bacteriology (Holt *et al*., 1994).

9. **PATHOGENICITY TEST**

Carrot-disk and potato-disk bioassays can be used for pathogenicity testing.

Carrot and potato-disc bioassays: Carrot (*Daucas carota*) discs were used (Aysan *et al*., 2003). Carrot will be cut into small discs, properly washed with 95% of Bleach solution for 3 min, and then washed with double distilled water. Sterilized filter paper will be placed on each Petri dish where the carrot disc is to be placed. A single colony of bacterial culture was picked from petri plates and poured into each disc present in the Petri dish. Subsequently, these plates were kept in an incubator for 20 d to observe the formation of young galls on the carrot disc. The same method of carrot disc bioassay was repeated for potato discs (Hussain *et al*., 2007).

**10. EPIDEMIOLOGY**

Any condition that creates wounds or injuries to the root crown increases the risk of infection and subsequent disease development. Once the host tissue is transformed by the infection processes, galls remain even in the absence of the bacterium. The extent of crown gall damage to grapes depends greatly on a number of factors, such as the site of the gall (trunk, root, or cane), when infection occurs (planting time or older plants), and region. In cold, humid areas, the disease can be devastating because freezing causes numerous galls to form on canes and trunks, often leading to the death of the plants, which must remain moist for several hours for infection to occur (Narratives).

**Survival:** Agrobacterium tumefaciens survives in the infected host tissue but also in the rhizospheres of various plants and is likely to be a soil saprophyte. It is found in both native and cultivated soils. The disease is uncommon in warm areas of the tropics (narratives). At a temperature of 20-25°C the incubation period is of 13-14 days, whereas at a lower temperature of 10-15°C are necessary 27-28 days for bacteria cell incubation. infection potential is increased by a higher relative humidity of 80-90%, and decreased by light intensity. The disease is also favored by wet and compact soils, frost damage to plants, nitrogen fertilizers, low affinity between scion and rootstock, injury produced by hail, and attack of nematodes (Vizitiu *et al*,2012). At an optimum growth temperature of 25–28 °C, bacteria metabolize a wide range of mono- and disaccharides and salts of organic acids.

**9. ECONOMIC IMPORTANCE**

Crown galls can affect a wide range of crops, including woody ornamentals, tree fruits, and small fruits. A few vegetable crops and herbaceous ornamentals are also susceptible; however, these crops are only occasionally affected. Crown galls can cause losses in landscapes, nurseries, orchards, and vineyards.About 80% of domestic cut roses are grown hydroponically. Commercial rose cultivars that use cutting for production suffer from crown gall disease (Lim 2023**).**

**10. Control measures**

* 1. **Cultural methods**

Grapevine propagation material must be provided from healthy mother plantations;

Avoid setting up plantations in soil infected with virulent *Agrobacterium* spp. and/or nematodes; do not establish a new plantation in clay soils, with poor drainage, in cold areas, wet and northern exposure, or with low nutrient or organic matter (Vizitiu *et al.,*2012)

Prevent the introduction of bacteria by planting only disease-free nursery stock. Remove and destroy all the infected materials. In addition, they do not grow susceptible plants in soils previously infested with the pathogen. Avoid wounding susceptible plants and control chewing insects and other pests that cause injuries. If possible, a budding technique should be used rather than grafting during vegetative propagation.

Disinfest cutting tools to reduce plant-to-plant transmission of the crown gall bacterium and other pathogens.

The most important control practices are sanitation, such as storing plants in clean facilities and rotation of fields to prevent the buildup of inocula. In nurseries, methods have been adopted to minimize the spread of pathogens and infection.

Hydroponic aubergines grown on perlite have been shown to develop fewer symptoms of HRD than rockwool (Bosmans *et al.*,2017).

* 1. **Mechanical methods**
* Vizitiu *et al.* (2012) recommended the following mechanical and physical control methods:
* Disinfection of canes before storage;
* The planting material (scions and rootstocks) used for multiplication can be treated before grafting by immersion in hot water at a temperature of 50- 52°C, for 30-60 minutes
* Use frost resistant grapevine varieties for the new vineyards;
* Strong recommendation is crop rotation in the vine nursery;
* Before planting, vines must be carefully selected, sorted, and excluded from infected plants, and to ensure good plant nutrition, it is recommended to supply the soil with nutrients and lime to avoid vine stress due to poor nutrition or low pH.
* After planting is important to avoid mechanical injury of the plants, the winter period is recommended to protect the trunks against frost, because any injury of the trunk as a result of the cold effect represents a gateway for bacterial entrance.
* Avoid supplementary nitrogen fertilization as much as possible because it could represent a food source for pathogenic bacterial cells.
* Supplementary potassium fertilization is recommended to improve vine resistance to cold and to obtain better resistance of the canes to virulent species of *Agrobacterium*
* Use a double or multiple trunk system for training. This system may be useful for minimizing losses due to crown galls; if one trunk is infected, it can be removed. The remaining trunk could be pruned, leaving a full number of buds until the second trunk could be renewed.
* Adopt a low or high management forms on the arms with periodic replacement;
* Avoid a prolong vegetation which is detrimental to cane maturation;
* Burying the mature grapevines canes for the winter period to avoid injury due to frost; Treat the soil for nematodes presence; the nematodes injure the roots and stems of the grapevines and in the same time favor the penetration of bacteria into plant tissues; Apply specific treatment to kill all larvae and insects with chewing device, because they are passive carriers of the bacterium;
* Avoid cold water irrigation.
* Avoid plants mechanical injury during cultural practices;
* Remove the infected plants from nurseries and mother plantations;
* Diseased plant material will be collected and placed into sealed packages to prevent the spread of infection to other plants or the surrounding soil.
* All infected plants, or their debris will be burned;
* Weed control by mechanical work or/and with total herbicides is strongly recommended;
* Surgical excision of crown gall tumors from infected plants is ineffective in controlling this disease.
  1. **Chemical methods**

At a concentration of 200 mg/ml, peppermint essential oil completely inhibited tumor formation in tomato plants inoculated with the pathogenic strain A. tumefaciens (Hsouna *et al*., 2019).

Vizitiu *et al.*(2012) studied *Agrobacterium* pathogen infection in grapevines and proved an ineffective effect of chemical compounds on bacterial cells inside plant tissues, but beneficial effects could be obtained by using different chemical solutions for the treatment of infected soil. Acrolein (2-propenal) has been formulated and registered as an aquatic herbicide in irrigation systems. This product was proven to have an efficient effect on the control of A. tumefaciens in the soil. They are also used to control microorganisms and bacteria in oil wells, liquid hydrocarbon fuels, cooling water towers, and water treatment ponds. Pu and Goodman (2011) studied the interaction between plants and pathogens in vineyards established with indexed Agrobacterium-free grapevine plants, but in Agrobacterium-infested vineyard soil. After 16 months, bacteria were detected in grapevine plants. In early spring, when the sap began to flow into the trunk, a high level of sap infection was detected, indicating that the primary source of pathogens was the soil. Therefore, we tested the influence of fumigation of soil with Vorlex. Repeated analysis with the same trunks showed a decrease in the initial infection level and a lower frequency of tumor development as a result of fumigation.

Mud for sink vine roots before planting will be prepared with fungicide that provides protection against infections with bacteria, for example, copper sulfate 1%, captadin 50 PU 1% Topas EC-0,025% or Kasumin, Potassium salt 0,5%, Rovral, Mikal, Saprol in higher doses than to prevent fungal pathogens.

All the equipment and tools used for cutting and forcing the grapevines will be disinfected with formalin 2-5%, sodium hypochlorite 1-3%, before and during working; disinfection of canes before storage.

The planting material (scions and rootstocks) used for multiplication could be treated before grafting by: a) bathing for 15 minutes in formalin solution 0,3-1%; c) spraying or bathing with Chinosol W 0,5% or Solvochim 0,5%; d) dipping for 10-15 minutes in Captan 0,2% or copper sulfate 1% solution; x

The soil has to be disinfected by steam; 2% formalin (10 l/m², especially in greenhouses) or leave gaps in the plantations for at least 3 years, the same procedure is applied to soil in greenhouses, with steam (82˚C for at least 30 min) or fumigants after removing all plant material. For soil fumigants, all the manufacturers’ directions and precautions were carefully followed.

From May to August are recommended treatments with products based on copper, such as Turdacupral 50 PU 0,4 %; Funguran OH 50 WP 0,3%; Champion 50 PU 0,3%; Captadin 50 PU 0,2%; Captan 50WP 0,2% - to stop the proliferation of bacteria.

Cationic surfactants, such as benzalkonium chloride (BC), cetyltrimethylammonium bromide (CTAB), and Physan 20, a quaternary ammonium compound (a mixture of alkyl dimethyl benzyl ammonium chloride and alkyl dimethyl ethyl benzyl ammonium chloride), have been shown to eliminate 100% of tumorigenic agrobacteria in water suspensions of hydroponics treated at 7, 5, and 2 ppm (Bosmans *et al.*,2017).

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* 1. **Biological methods**

Some strains of *A. tumefaciens* are sensitive to agrocin antibiotic produced by *A. radiobacter*, a closely related bacterium that does not infect plants. Given that *A. radiobacter* produces an analog of the opine, agrocinopine A (Agrocin84), which inhibits DNA replication and bacterial cell growth of *A. tumefaciens*, this feature was used for biological control. Therefore, a 1:1 ratio of *A. tumefaciens* and *A. radiobacter* strain K84 suspended in water was used to treat seeds, seedlings, or cuttings before planting. Agrocin-84 acts only as a preventative treatment to protect wound sites against pathogenic invasion and does not cure bacterial infections. In recent years, has proposed utilization of non-pathogenic *A. vitis* strain F2/5 has been used for the biological control of virulent strains. This strain, such as *Rhizobium leguminosarum bv trifolii*, produces an antibiotic (trifolitoxin-TFX) that is toxic to many *A. vitis* strains *in vitro*, reducing the number of galas, their size, and, in some cases, killing pathogenic bacteria. Nonpathogenic *A. vitis* strains F2/5 may be applied to the injured tissues of grapevines to prevent the appearance of crown galls. Another nonpathogenic strain of *A. vitis* (VAR03-1) was used by Kawaguchi et al. as a biological control agent against the crown galls of grapevine plants. According to their data, by applying a 1:1 ratio of pathogen/non-pathogenic strain suspension in tomatoes, sunflowers, and vines, a lower incidence of tumors was obtained. Strain HX2 of *Rahnella aquatilis* has been reported as a potential biological control agent for the crown gall of grapevines. Antibacterial substances produced by this strain have bactericidal effects against the virulent strain of *A. vitis*, both in vitro and in vivo. *Rahnella aquatilis* HX2 was isolated from soil samples and was demonstrated to have a significant inhibitory effect on tumor growth in grapevines. By immersing the basal ends of grape cuttings in an HX2 cell suspension, inhibition or complete prevention of crown gall formation in plant material artificially infected with the virulent strain *A. vitis* K308. Further studies in vineyards revealed normal plant growth and no microflora degradation in the soil as a result of HX2 cell suspension treatment. For the control of A*. tumefaciens* pathogen were tested biological preparations of paurin and tumarin were obtained from *Pseudomonas fluorescens* cultures. Before planting, vines are dipped in solutions of paurin and tumarin for 10-15 minutes, or their roots are sprayed with these biological compounds to prevent further infection (Vizitia *et al*.,2012).

Biological control methods were used to protect plants from possible infections with *Agrobacterium tumefaciens* Galltrol-A, Nogall, Diegall, and Norbac 84C before planting.

Utilization products based on *Bacillus subtilis* for the disinfection of scions and rootstock strings for the production of grafted vines

Careful disinfection of spaces for grafting and forcing, tools used for cutting, or soil grinding in vine nurseries must also be disinfected periodically to prevent the infection of healthy plants.

For the commercial [biological control agents](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/biological-control-agents), endophyte *B. subtilis* SR63 were the most effective strains against *Agrobacterium* sp., and was identified as a useful biocontrol agent for the management of crown gall disease (Ferrigo *et al*., 2017).

Dip cutting or treatment of fresh wounds with a suspension of the biocontrol agent *A. radiobacter* (strain 84). This bacterium is established on plants and produces a compound that is toxic to most strains of the crown gall bacterium.

Biological control strains of *Agrobacterium radiobacter*, K-84 or K-1026, the latter being a genetically engineered strain of K-84, are frequently used to treat seeds and plants to be planted in nurseries and fields. Biocontrol strains multiply in wounds and prevent gall formation if applied soon after a wound occurs. However, there are tumorigenic strains of *Agrobacterium* spp. that are not controlled by biocontrol strains at prescribed concentrations. For control of some strains, experiments in field and greenhouse studies indicated that the ratio of cell density of the biocontrol agent to the pathogen were effective when the number of cells were around 1,000:1(Agrios,2005).

Excellent biological control of crown galls is achieved by soaking germinated seeds or dipping nursery seedlings or rootstocks in a suspension of a particular strain (No. 84) of *Agrobacterium radiobacter*. This bacterial strain is antagonistic to most strains of *A. tumefaciens*. Some controls were also obtained by treating non-germinated seeds with an antagonist or by drenching the soil with a suspension of the antagonistic bacterium. It is postulated that the antagonist controls crown gall initiation by establishing itself on the surface of the plant tissues, where it produces the bacteriocin agrocin 84. This bacteriocin inhibits most virulent *A. tumefaciens* strains. Unfortunately, some strains of *A. tumefaciens* inherited from strain 84 are resistant to agrocin 84. Therefore, a new strain (K-1026) is now being used because it lacks the ability to transfer its resistance gene to pathogenic *Agrobacterium* strains.

Prophylactic measures using antagonistic soil-borne bacteria, such as *A. radiobacter*, which harbors the plasmid pAgK84 encoding the antibiotic bacteriocin K84, have been successful only for certain strains of *A. tumefaciens*. Therefore, the strain specificity of biological control agents limits their use in sensitive pathogen strains. Other prophylactic strategies include maintaining the propagation of nurseries free of crown gall-affected plants and sanitary culturing practices. However, the future of crown gall control in agronomically important plants lies in the use of genetic engineering technologies to produce *Agrobacterium*-resistant lines of fruit and nut trees, including grapevines and canes (Agrios,2005).

Agrocin 84 prevents protein synthesis by inhibiting the essential enzyme leucyl tRNA synthetase

Kerr and Bullard (2020) reported that the genes for the synthesis of agrocin 84 are located on a plasmid, pAgK84, a conjugative plasmid that can be transferred to other strains, including pathogens; it also carries immunity to agrocin 84. Studies indicate that K84 also produces agrocin 434, which is active against *A. rhizogenes* and causes hairy root disease in hydroponic tomatoes (Bosmans *et al.*,2017). Gargouri *et al.* (2017) reported that *Bacillus* spp. has the potential to reduce the growth of *Agrobacterium* in tomatoes.

* 1. **Resistant/Tolerant varieties (if any)**

Cut rose varieties Violettea and Ocean Song', the parental line of ‘Violetta’ was found to be relatively resistant probably due to inherited resistance (Lim, 2023)

Vizitiu *et al.* (2012) suggested avoiding the establishment of vineyards with varieties susceptible to crown galls, such asAfuz Ali’, ‘Ceaus roz,’ ‘Ceaus roúu,’’ Italia, and Merlot.’

Use for the establishment of new vineyards less susceptible cultivars to crown gall, such as: ‘Fetească regala,’ ‘Furmint,’ ‘Coarna neagra,’ ‘Pinot gris’

Use also as rootstocks only resistant or less susceptible varieties to crown gall, such as: ‘Riparia Gloire,’ and ‘C 3309. ’ These rootstocks do not prevent infection but are resistant to transformation. Rootstocks can greatly affect the severity of crown gall infection caused by grapevines.

Bosmans *et al.*(2017)studied three large clusters in the genus *Agrobacterium* (Table 2) corresponding to biovars 1, 2, and 3. Biovars are determined by chromosomal genes and not plasmids, and therefore, better reflect phylogenetic relationships. As a result, tumorigenic, rhizogenic, and non-pathogenic strains can be found within the same biovar. More specifically, biovar 1 contained strains of *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter*, including the type strains of *A. tumefaciens* and *A. radiobacter*. Biovar 2 also contained strains of all three species, including the type strain of *A. rhizogenes*. Biovar 3 contained *A. tumefaciens* and *A. vitis* strains.

Abd-El-Aziz *et al.* (2021) revealed that the inhibition zone produced by the antagonist *A. radiobacter* against *A. tumefaciens* 27AS\_ Pp4 was 11.8 mm in diameter, whereas it was 10.2 and 11.0 mm in caraway and thyme essential oils, respectively.In case of pot culture average number of galls were less in these oil treated apricot plants when compared to control(Table 3). In case of thyme oil treated apricot plants only 0.4 number of galls appeared and in caraway oil treated apricot plants 1.4 number of galls were developed indicating the efficacy of these oils.

**Table 3: Efficacy of caraway and thyme oils on *A.tumefaciens* of apricot (modified from Abd-el Aziz *et al*. *,*2021)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Treatment** | **Average number of galls/plant** | **Average weight of galls/plant(g)** | |
| **Fresh** | **Dry** |
| Caraway | 1.4 | 0.26 | 0.12 |
| Thyme | 0.4 | 0.07 | 0.03 |
| *A.radiobacter* | 0.0 | 0.0 | 0.0 |
| Control | 3.8 | 0.52 | 0.25 |

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Crown gall on rose. (Photograph courtesy of Dr. Powelson, Oregon State University)

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