

## RESEARCH ARTICLE

## MOLECULAR, BIOCHEMICAL AND BIOLOGICAL DIAGNOSIS OF *AGROBACTERIUM TUMEFACIENS* CAUSING APPLE CROWN GALL DISEASE

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## ARTICLE DETAILS

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## ABSTRACT

The research aimed to identify and characterize crown gall pathogen that affects apple trees planted in Nineveh and Dohuk governorates. Based on its biochemical, and molecular characteristics, *Agrobacterium tumefaciens* colonies appeared yellow-green, on D1 Agar, on nutrient agar colonies were creamy white, smooth, convex, round, and regular margins emerged. The colony's color darkens and turns gelatinous with age. *A. tumefaciens* are Gram-negative and showed positive results for the generation of catalase, urea, indole, 3-ketolactose, citrate utilization, motility, carbohydrate fermentation, KOH solubility, growth at 35 °C, and growth with 2% NaCl, *A. tumefaciens* infection was observed. By developing gall on apple seedlings cultivated. In vitro, *A. tumefaciens* demonstrated its capacity to grow tumors on carrot discs. When infected tobacco leaves, it simultaneously produced a favorable effect, demonstrating the responsiveness of tobacco leaves to it. Furthermore, *A. tumefaciens* isolates were used to inoculate tomato plants, which is thought to be a crucial diagnostic technique for identifying the source of crown gall disease.

## KEYWORDS

*Agrobacterium tumefaciens*, crown gall, bacterial disease, Biochemical test, pathogenicity.

## 1. INTRODUCTION

Apple *Malus domestica* Borkh. belongs to Rosacea. It originated in the South Caucasus region and has been found in East Asia for thousands of years. Apple fruits have high nutritional and economic value (Colavita et al., 2021). Many countries have been interested in cultivating it and expanding the cultivated areas, which reached 95,835,970 (Hannah et al., 2023). Crown gall is one of the oldest known bacterial diseases of plants, caused by *A. tumefaciens*, infecting more than 93 families, including 600 species of dicotyledonous plants (Kado, 2014) the disease is a devastating bacterial disease affecting orchards, frequently occurring in nurseries and orchards growing rose plants such as apple, cherry, peach, pear, and rose, as well as various types of nut trees such as walnut and pecan, and also in grapevines (Asghari et al., 2020, Necreală et al., 2021, Sharma., 2022). During the infection process, uncontrolled tissue growth at the site of infection may lead to the infestation of plant pathogen. Infected plants suffer from general weakness and reduced leaf size, especially when seedlings or young cuttings are infected in the early stages of their growth. In severe cases of infection, it may lead to the death of branches or even the death of the entire plant. Crown gall is caused by strains of root-tumor-causing bacteria, including several species of the genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium*. *A. tumefaciens* is the most common strain of bacteria causing crown gall. These bacteria are known to be soilborne microorganisms, and are particularly dangerous in sandy or sandy loam soils. They can also remain dormant in plants without showing any symptoms of disease until favorable conditions for infection, such as wounds, are present (Al-Tememe et al., 2022 and Moura et al., 2022). *Agrobacterium* is often transmitted through asymptomatic seedling or trees, which may cause the disease to appear in the trees later. (Oksel et al., 2024).

## 2. MATERIAL AND METHOD

## 2.1 Sample collection

Samples were collected from apple trees which showed crown gall symptoms in Nineveh and Dohuk Governorates orchards. fresh galls were cut from infected branches and brought to the Plant Pathology Laboratory in the Plant Protection Department for pathogen isolation.

## 2.2 Isolation

Bacteria were isolated from gall which was superficially sterilized with 1% NaOCl solution for 20 min, washed three times with sterile distilled water, crushed using a sterile ceramic mortar with a little NaCl (0.9%) sterile normal saline solution, and incubated at 28°C for 30 min, loop full from incubated solution was taken to inoculate D1 agar plates and incubated at 28 °C for 24 h. a loop full from a single colony streaked onto nutrient agar, incubated at 28 °C for 24 h. (Oh, 2014).

## 2.3 Identification test

## 2.3.1 Biochemical Identification

The following Biochemical tests were performed to identify bacteria isolated from gall. Gram Staining, catalase testing, citrate utilization, indole production, carbohydrate fermentation, gelatin liquefaction, KOH solubility, growth at 35 °C, growth with 2% NaCl, urea enzyme production, starch hydrolysis, and 3-ketolactose (Green and Goldman, 2021).

## 2.3.2 Molecular Identification

## 2.3.2.1 DNA Extraction:

DNA was extracted from bacterial samples using the ZR Fungal/Bacterial DNA MiniPrep™ Kit (D6005) according to the user manual included with the kit. The concentration of the extracted DNA was then measured to check its efficiency.

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### 2.3.2.2 Primers :The bacterial specific primers (16S rRNA gene) were used:

Forward primer: 5'-GTG TAG CCG TGA AAT GCG-3'

Reverse primer: 5'-ACG GGC GGT GTG TAC AA-3'

### 2.3.2.3 Polymerase Chain Reaction (PCR):

The target genes were amplified using PCR. Reaction mixtures were prepared using the Maxime PCR PreMix Kit (i-Taq), with the optimum reaction conditions set as follows: Initial Denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, Annealing at 56°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The gel was prepared by melting the agarose in 1X TBE Buffer, then pouring it into a mold containing a comb to create sample holes. Samples were added with 6X Loading Dye to the gel, and the analysis was run at 7 V/cm for 1–2 h. DNA was detected using Red Safe Staining Solution under UV light. PCR products were compared with the 1000 Plus bp Ladder to determine the product sizes and confirm that they matched the expected sizes. The results showed clear bands indicating successful amplification of the target genes of the bacterial isolates.

### 2.3.3 biological diagnosis

#### 2.3.3.1 Gall formation on tomato.

Tomato seedlings *Solanum lycopersicum* L. in the four-leaf stage, were inoculated in the seedlings stem with 50 µl of bacterial suspension by making three pricks using a sterile syringe. Distilled water was used as a control. Tomato seedlings were kept in the greenhouse until the appearance of tumors (Kuzmanović et. al., 2011; Mashiguchi, et al., 2019).

#### 2.3.3.2 Gall formation on carrot disc.

Inoculum was prepared by taking a bacterial inoculum from a solid medium and inoculating it with Luria Bertani Broth (LBB) in test tubes and incubating at 28 °C for 48 hours. the final optical density ranged between

0.1 - 1.5. at 600 nm, carrot disks were sterilized with a 2% sodium hypochlorite solution and 70% ethyl alcohol for 10 minutes, washed with sterile distilled water, dried by placed between sterile filter papers, the disks were stabbed using a sterilized needle containing a bacterial inoculum, then the discs were transferred to sterile Petri dishes containing Water Agar medium. incubated for 16 hours of light and 8 hours of darkness. carrot discs were examined periodically to detect tumors formation (Ferdous et al., 2021).

#### 2.3.3.3 Tobacco sensitivity

Tobacco seedlings *Nicotiana tabacum* at four leaf stages were inoculated by the Leaf infiltration method by injecting 1 ml of bacterial inoculum into the intercellular space of tobacco leaves using a Microsyringe with a needle 28 gauge. The inoculated plants were placed in a greenhouse, covered with polyethylene bags to maintain their humidity, and monitored until symptoms appeared, a control treatment seedlings were injected with 1 ml of sterile distilled water (Gutsch et al., 2023).

#### 2.3.3.4 Gall formation on apple seedling

One-year-old apple seedlings of the Ginger Cold variety were Inoculated by cutting the main stem above the grafting area, transferring a disc of bacteria culture grown on Luria Bertani agar for 24 h, and tying the area with parafilm to conserve moisture seedlings were kept in the plastic greenhouse canopy until the appearance of tumors (Rouhrazi and Rahimian, 2014) .

## 3. RESULTS

### 3.1 Isolation

Isolation results from crown gall on apple tree stems showed the appearance of yellowish-green colonies on D1 Agar. Single colonies on nutrient agar were cream in color, smooth, convex, and circular with regular edges. As the colony ages, its color darkens and becomes sticky and gelatinous (Figure 1).

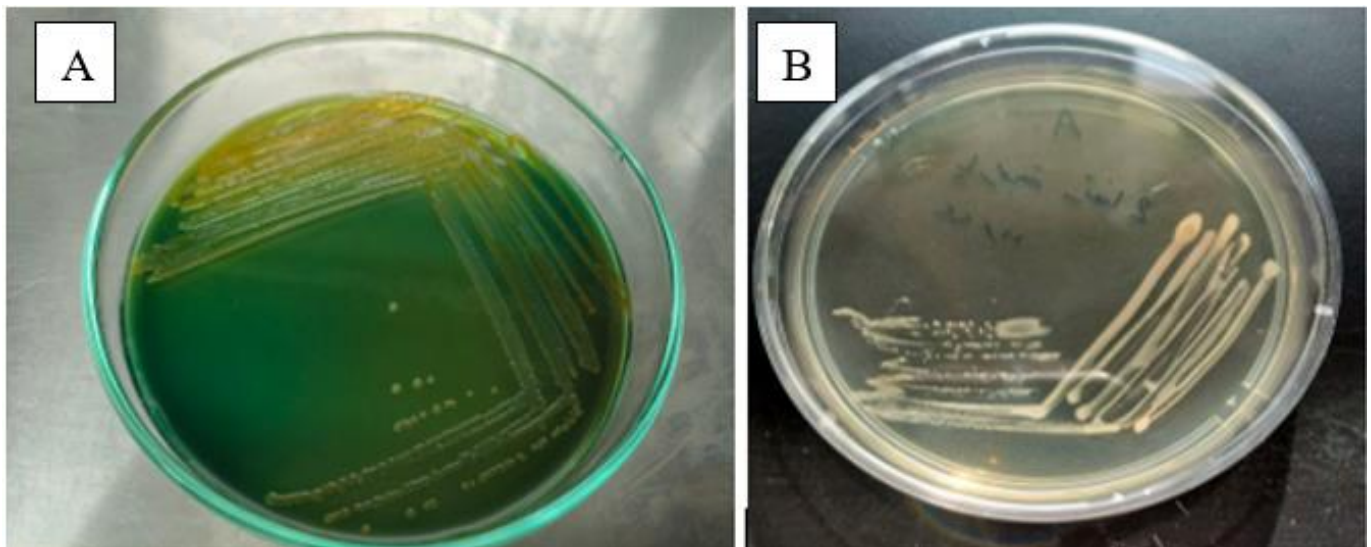


Figure 1: *Agrobacterium tumefaciens* on : A- D1 agar . B- Nutrient agar.

### 3.2 Identification Test

#### 3.2.1 Biochemical Identification

Morphological and Biochemical test results were positive except starch hydrolysis test and Gram stain test , as shown in Table 1. and Table 2.

Table 1: Morphological and Physiological characteristics of *Agrobacterium tumefaciens* isolates

Test	At.1	At.2	At.3	At.4	At.5
Gram stain	-	-	-	-	-
Motility test	+	+	+	+	+
Growth at 35 °C	+	+	+	+	+
Growth on PDA	+	+	+	+	+
Growth with 2% NaCl	+	+	+	+	+
KOH solubility	+	+	+	+	+

- (At.) *Agrobacterium tumefaciens*. (+) Positive result. (-) Negative result.

**Table 2:** Biochemical characteristics of *Agrobacterium tumefaciens* isolates

Test	At.1	At.2	At.3	At.4	At.5
Catalase production	+	+	+	+	+
Urease production	+	+	+	+	+
Indole production	+	+	+	+	+
3-Ketolactose production	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Carbohydrate fermentation	+	+	+	+	+
Gelatin liquefaction	-	+	-	+	+
Starch hydrolysis	-	-	-	-	-

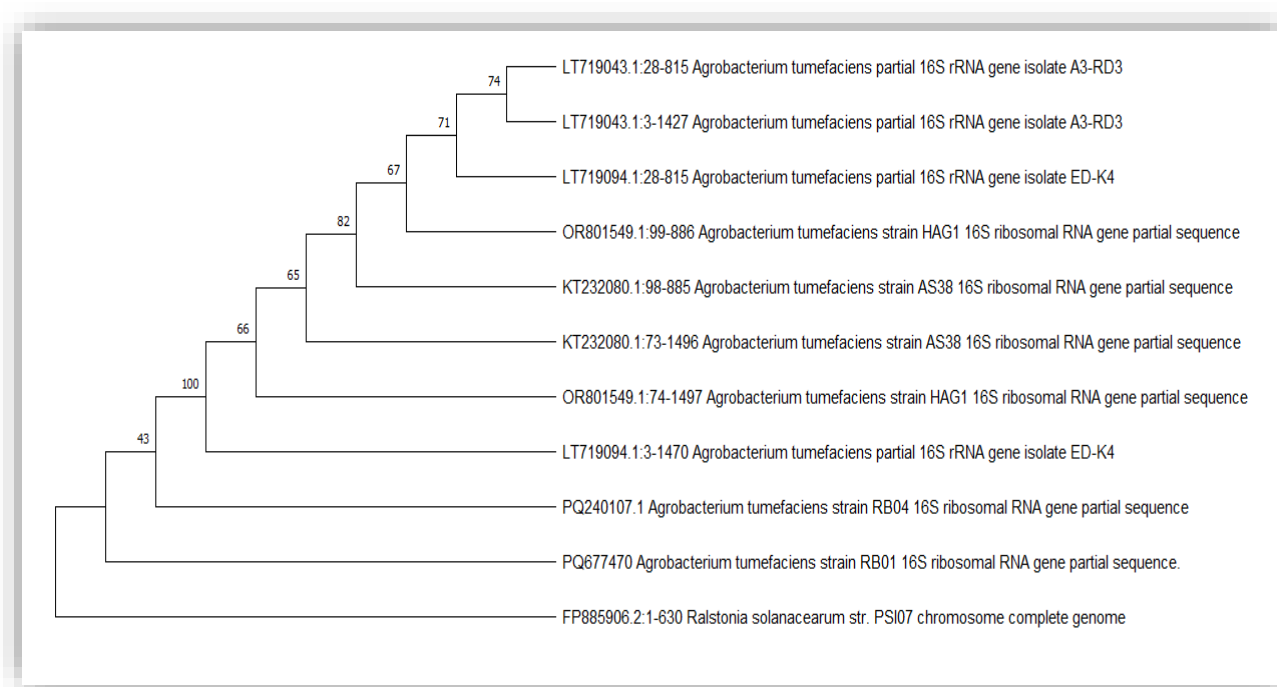
- (At.) *Agrobacterium tumefaciens*. (+) Positive result. (-) Negative result.

### 3.2.2 Molecular Identification

Two *A. tumefaciens* isolates isolated from apple orchards in Mosul and Duhok cities were identified and registered in the National Center for Biotechnology Information with accession numbers PQ677470.1 and PQ240107.1 for the isolates, respectively.

The phylogenetic tree (Figure 2) shows a common ancestry among *A. tumefaciens* isolates and high values at some branches indicate the reliability of the phylogenetic relationships represented. The phylogenetic tree also shows that strains PQ677470.1 and PQ240107.1 are closely related and strain PQ677470 appears as a single branch and is closer to strain PQ240107.1 than to the other strains in the tree. The relationship between this strain and neighboring branches is supported by a low

Bootstrap value of 44, indicating that the statistical support for this relationship is relatively weak compared to other branches with higher support values. Strain PQ240107.1 is very closely related to strain PQ677470, reflecting a high similarity between their gene sequences. The relationship between the sequences for this strain is very reliable as shown by the high Bootstrap values of 100, indicating the accuracy of the evolutionary relationship between the different partial sequences of the same strain. The two strains show: High genetic similarity: The two strains are very close in the phylogenetic tree and are very close evolutionarily within the same bacterial species *A. tumefaciens*. This suggests that they may belong to two subgroups or sub strains within the same species, the difference between which may be due to slight differences in genetic sequence, or perhaps reflect differences in ecology or functional behavior.



**Figure 2:** The phylogenetic tree was constructed based on 16S rDNA sequences to illustrate the estimated relationships between *Agrobacterium tumefaciens* isolates and their closest related strains from the NCBI GenBank database. The tree was constructed using the nearest-neighbor-joining method and validated using the bootstrap method with 1000 iterations.

### 3.2.3 biological diagnosis

#### 3.2.3.1 Gall formation on tomato.

Gall appeared along tomato seedlings stem inoculated with *A. tumefaciens* after 18 days, and the symptoms increased with time (Figure 3 - a ).

#### 3.2.3.2 Gall formation on carrot disc.

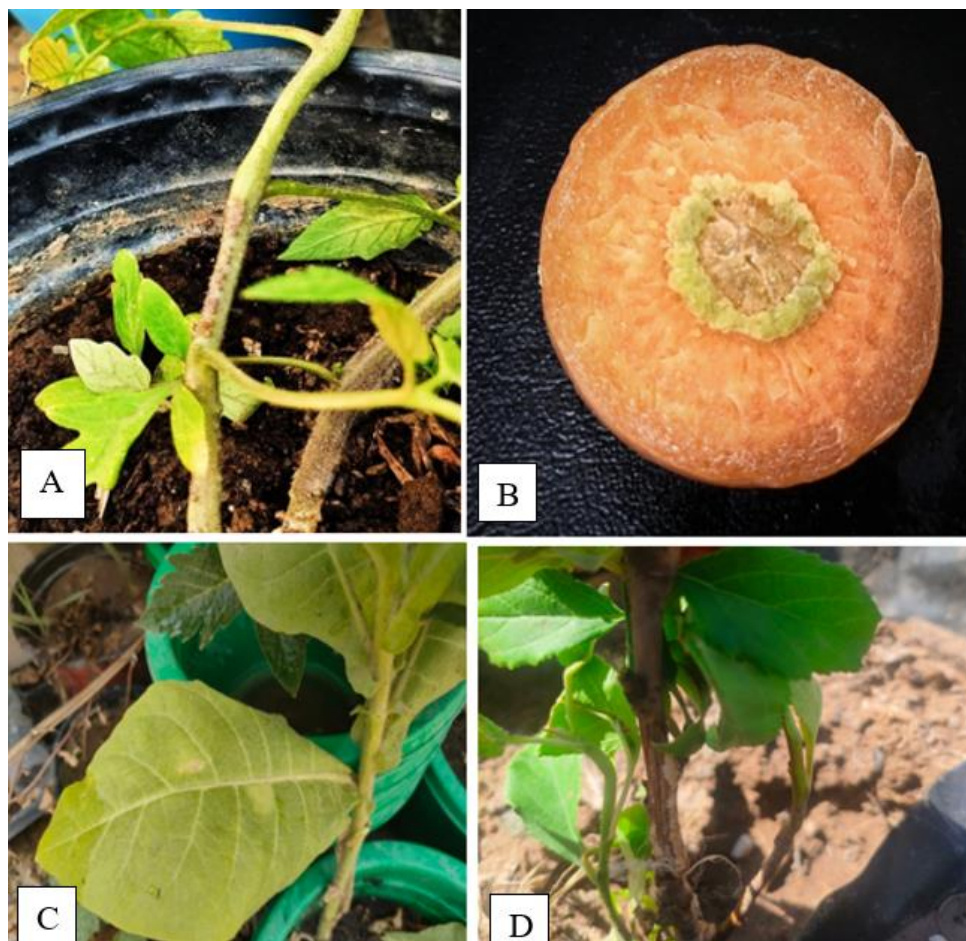
*A. tumefaciens* was able to cause green tumors on carrot disc surfaces after 21 days, (Figure 3 - b).

#### 3.2.3.3 Tobacco sensitivity

sensitivity test results on tobacco leaves showed that *A. tumefaciens* isolates obtained from apple trees' crown gall were able to cause yellow spots to appear in the inoculated area, later turned to light brown (Figure 3-c). The bacterial isolates varied in the speed of appearance of these symptoms.

#### 3.2.3.4 Gall formation on apple seedling

Apple seedlings inoculated with *A. tumefaciens* showed the symptoms of tuberculosis after 21 days above the grafting area, and the size of these galls increased over time (Figure 3 - d).



**Figure 3:** Grown gall symptoms on: A- Tomato (*Solanum lycopersicum* L.). B- Carrot disc (*Daucus carota*). C- Hypersensitivity on Tobacco plant. D- Apple seedling.

#### 4. DISCUSSION

Crown gall caused by *A. tumefaciens* frequent disease in Dicotyledonous plants, such as numerous woody shrubs like apples, peaches, grapes, roses, and different herbaceous plants (Sharma, 2022), Galls are often located in the crown region at or below the soil's surface (Kado, 2014)

*A. tumefaciens* is a gram-negative bacteria that grows pink to red on Yeast extract mannitol agar supplemented with Congo red (Alheyalee and Ibrahim, 2025). In this investigation, every isolate developed pink to red colonies on YEMA that had been enhanced with Congo red, suggesting that they had absorbed the dye quite well. All of the isolates stained pink when subjected to Gram staining, demonstrating that they are Gram-negative bacteria. (Kariuki, 2020).

The identification of bacteria was made through various tests, including catalase, Potato Dextrose Agar (PDA), and urease tests. All isolates showed positive results, indicating their growth and usage of citrate. The presence of sodium chloride and citrate did not affect their growth, indicating their acid and alkaline production. Benedict's reagent caused a yellow ring of copper oxide (I) precipitate to form at the bottom of the test tube in the 3-ketolactose test. The differentiation of *A. tumefaciens* was aided by this test. The 3-ketolactose test was another method used to discriminate between *Agrobacterium* and *Rhizobium* species by (Oh, 2014).

*A. tumefaciens* affects apple and tomato seedlings after 18 - 21 days of artificial inoculation. Symptoms include small, milky white, soft callus galls that harden and become dark. Crown galls form due to increased divisions, leading to increased cell number and size. This pressure on surrounding tissues and obstruction of water and nutrient access is attributed to local accumulations of plant hormones synthesized by *A. tumefaciens* (Ramos, et al., 2012). The bacteria secrete the growth regulator IAA, which stimulates parenchymal cells to divide and form tumors or galls. Growth regulators play a role in plant pathogenicity (Dodueva, et al., 2020).

#### REFERENCES

Alheyalee, R. S., Mahmood, and B. Y., Ibrahim, 2025. Evaluating the Influence of Chitosan, Cinnamic Acid, and Tannic Acid on *Agrobacterium tumefaciens* Growth and Biofilm Formation. IOP Conf. Ser.: Earth Environ. Sci. 1449 012050.

Al-Tememe, Z. , Al-Hakeem, A. , Baqir, H. and Fadel, N., 2021. The first register and Molecularly diagnosis of *Agrobacterium tumefaciens* that causes crown gall disease on *Ficus* sp in the provinces of Karbala and Babylon/ Iraq. International Journal of Agricultural and Statistics Sciences. 16. Pp. 1845-1847.

Asghari, S., Harighi, B., Ashengroph, M., Clement, C., Aziz, A., Esmaeel, Q., and Ait Barka, E., 2020. Induction of systemic resistance to *Agrobacterium tumefaciens* by endophytic bacteria in grapevine. Plant pathology, 69(5), Pp. 827-837.

Colavita, G. M., Curetti, M., Raffo, D., Sosa, M. C., and Vita, L. I., 2021. Apple In Temperate Fruits (pp. 1-106). Apple Academic Press.

Dodueva, I. E., Lebedeva, M. A., Kuznetsova, K. A., Gancheva, M. S., Paponova, S. S., and Lutova, L. L., 2020. Plant tumors: a hundred years of study. Planta, 251, Pp. 1-28.

Ferdous, M. L., Hossain, M. N., Ali, M. O., Islam, M. S., and Yasmin, S., 2021. Morphological, biochemical and molecular identification of the wild strain of *Agrobacterium tumefaciens* from crown gall infected mango tree. Fundamental and Applied Agriculture, 6(1), Pp. 43-49.

Green, L. H., and Goldman, E. (Eds.), 2021. Practical handbook of microbiology. CRC press.

Gutsch, A., Berni, R., Hausman, J. F., Sutera, F. M., Dehsorkhi, A., Torabi-Pour, N., and Guerriero, G., 2023. A Study on the Use of the Phyto-Courier Technology in Tobacco Leaves Infected by *Agrobacterium tumefaciens*. International Journal of Molecular Sciences, 24(18), 14153.

Hannah, R. , Pablo R. and Max R., 2023. "Agricultural Production". Data adapted from Food and Agriculture Organization of the United Nations.

Kado, C. I., 2014. Historical account on gaining insights on the mechanism of crown gall tumorigenesis induced by *Agrobacterium tumefaciens*. Frontiers in microbiology, 5, 340.

Kariuki, N. A., 2020. Management of crown gall disease in selected rosa hybrida farms in kenya using artemisia annua leaves and zingiber officinale rhizome extracts (doctoral dissertation) kenyatta

- university. (4) Pp. 141-148.
- Kuzmanović, N., Ivanović, M., Čalić, A., Gašić, K., and Obradović, A., 2011. Differentiation of Phytopathogenic agrobacterium spp.. Pesticidi i fitomedicina, 26(3), Pp. 245-253.
- Mashiguchi, K., Hisano, H., Takeda-Kamiya, N., Takebayashi, Y., Ariizumi, T., Gao, Y., and Kasahara, H., 2019. Agrobacterium tumefaciens enhances biosynthesis of two distinct auxins in the formation of crown galls. Plant and Cell Physiology, 60(1), Pp. 29-37.
- Moura, A. B., Backhouse, D., de Souza Júnior, I. T., and Gomes, C. B., 2022. Soilborne pathogens. In Subsoil constraints for crop production (pp. 199-224). Cham: Springer International Publishing.
- Necreală, M., Rotaru, A., Chifan, R., Cărăbeț, A., and Ștef, R., 2021. Evaluation of bacterial pathogen Agrobacterium tumefaciens attack on some apple varieties. Research Journal of Agricultural Science, 53
- Oh, P. M., 2014. Comparison of two selective media for the isolation of Agrobacterium tumefaciens from the soil (Doctoral dissertation, UTAR).
- Oksel, C., Liyanapathiranage, P., Parajuli, M., Avin, F. A., Jennings, C., Simmons, T., and Baysal-Gurel, F., 2024. Evaluation of Chemical and Biological Products for Control of Crown Gall on Rose. Pathogens, 13(8), 708.
- Rouhrazi, K., and Rahimian, H., 2014. Biochemical and genetic characterization of Agrobacterium tumefaciens the causal agent of walnut crown gall disease in Iran. Archives of Phytopathology and Plant Protection, 47(20), Pp. 2493-2500.
- Sharma, N., 2022. Crown Gall of Stone Fruits-A Comprehensive review. Multi-Dimensional Aspects of Management, 172.

