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Microbial Contamination in Plant Tissue Culture and Elimination Strategies

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Abstract
The ability to establish and grow plant cell, organ, and tissue cultures has been widely exploited for basic and applied research, and for the commercial production of plants (micro-propagation). Regardless of whether the application is for research or commerce, it is essential that the cultures be established *in vitro* free of biological contamination and be maintained as aseptic cultures during manipulation, growth, and storage. Micro-organisms become problematic by virtue of their prolific growth under high nutrient *in vitro* conditions, hence the need to eliminate them from explants before entering the *in vitro* cycle. Exclusion of these organisms from potential plant tissue culture materials can be approached in several ways, and have been widely researched across different genera of crops. This review highlights the diverse sources of microbial contamination in plant tissue culture technology and the available elimination strategies.

Keywords: Plant tissue culture, in vitro, aseptic, contamination, microorganisms

Introduction

Micro-propagation, a plant tissue culture technique has emerged as one of the major components of plant biotechnology which has been applied in rapid clonal propagation, regeneration and multiplication of genetically manipulated superior clones, production of secondary metabolites and *ex-situ* conservation of valuable germplasms (Gupta and Ibaraki, 2006). *In vitro* propagation, a common method for vegetative propagation, is a plant tissue culture approach that has a number of advantages over traditional methods of plant propagation, chief of which is that exceptionally large numbers of plantlets can be produced in a very small amount of space and within a short time, especially for crops with long growth cycle (Ayalew *et al*., 2017).

Plant tissue culture, as an important tool has been widely employed in areas of agriculture, horticulture, forestry, pharmacy, and plant breeding. It is an applied biotechnology used for mass propagation, virus elimination, secondary metabolite production and *in vitro* cloning of plants (Oseni *et al*., 2018). The efficiency of the sterilizing techniques utilized on the explants prior to culture establishment is critical for tissue culture and surface sterilization of these explants (Singh, 2018). However, the success of plant tissue culture depends principally on the availability of a good protocol which will comprise effective steps for explant and media sterilization among others.

Microbial contamination in micro-propagation

The beginning and maintenance of viable *in vitro* cultures are complicated by microbial contamination. When it comes to plant pathogens, these contaminants are very harmful. When explants are obtained straight from field-grown plants, the problem is worsened. Microbial contamination refers to fungi or bacteria that are naturally present on the surface of an explant material, as well as natural apertures on the explant material that manifest after initiation and can be visible or latent. Growth media and plantlets contamination are the main causes of loss in large-scale micro-propagation facilities. While bacterial contamination of media can be controlled by autoclaving, the contamination of plantlets is very difficult to control, especially if the contaminants are endophytic (Tewelde *et al*., 2020). Sterilization of plant material and keeping the plantlets disease-free are the most important steps of the tissue culture protocol. In this process, an attempt is made to eliminate microbial contaminants from the surface and interior of plant materials, and giving the explant a fighting chance at survival *in vitro*. The success of tissue culture thus depends on the effectiveness of the sterilization methods used on the explants prior to culture initiation. All the materials used in the plant tissue culture must be sterilized to kill the microorganisms that are present by using appropriate sterilizing agents (Sessou *et al*., 2020).

Microbial contamination is a common occurrence in

plant tissue culture. Microbial contamination could come from explant-borne pathogen or as a laboratory contamination. Various efforts have been made to get rid of microbial contaminants in plant tissue culture (Medjemem *et al*., 2016). Surface sterilization of the explant to get rid of epiphytic microorganisms is carried out frequently. However, there are endophytic organisms which colonize inside of the living plant tissue and cause contamination of tissue cultures (Cassells, 2012). There are certain tissue cultures which take longer time for the regeneration of the plant, and if such tissues are bearing an endophytic organism, then it becomes difficult to maintain a contamination-free healthy tissue for longer duration (Nair *et al*., 2014). Even though it is possible to produce a large number of plants by micro-propagation, the greatest problem in this technique is contamination (Altan *et al*., 2010).

A wide range of microorganisms (filamentous fungi, yeasts, bacteria, viruses and viroids) and micro-arthropods (mites and thrips) have been identified as contaminants in plant tissue cultures, and according to Eziashi *et al.* (2014), bacterial contamination is a major threat in plant tissue culture. Plant tissue cultures could habour contaminants in a totally unsuspecting manner, either externally in the medium/plant or endophytically. Epiphytic bacteria may lodge in plant structures where disinfectants cannot reach during surface sterilization, while endophytic bacteria may be localized within the plant at cell junctions and the intercellular spaces of cortical parenchyma (Gunson and Spencer-Phillips, 1994). Bacterial contaminants found at explants initiation, already present in explants from time of collection, and resistant to surface disinfection are likely to be endophytic. Surface sterilization-resistant microorganisms may survive in the plant material for several subculture cycles and over extended periods of time without expressing symptoms in the tissue or visible signs in the medium (Eziashi *et al*., 2014).

Alla *et al*. (2013) reported effective sterilization of cassava nodes using Clorox (contains 5.25% NaOCl) at concentration of 20% for 15min. Yirrsaw *et al.* (2014) sterilized cassava nodal explants from greenhouse by exposing them to 0.1% NaOCl with 1–3 drops of Tween-20 for 10min after initial soaking in 70% ethyl alcohol for 1 minute, while Magaia (2015) reported the highest (87%) clean explants when nodal explants from greenhouse were exposed to 70% ethanol for 1 or 2 minutes followed by exposure to 0.05% Mercuric Chloride ($HgCl_2$) for 2 minutes or 0.1% $HgCl_2$ for 1 minute. Some of these findings were from concerted efforts by researchers to tackle the prominent challenge of culture losses due to microbial contamination.

Effect of microbial contamination on plant tissue culture

Contamination of plant tissue culture by different microorganisms such as bacteria and fungi reduces contaminated tissues productivity, and can completely prevent their *in vitro* growth. Several different methods are used to eliminate fungal and bacterial contamination, including the use of antibiotics and fungicides, as well as inactivation by heat and light (Sen *et al*., 2013). However, these methods can also be injurious to the *in vitro* tissue if not safely applied. Many sterilization agents are also toxic to the plant tissues, and hence optimum concentrations of sterilization agents, duration of exposure of explants to sterilization agents, the sequences of sterilization agents used among others, need to be determined to minimize explants injury and to achieve better survival (CPRI, 1992).

According to Bunn and Tan (2002), *in vitro* microbial contamination and control is one of the most important phenomena confronting the tissue culturist. With microorganisms now known to inhabit the inner tissues and intercellular spaces of a large number of plant species, the concept of "sterile culture" appears to be overstated. The inability to control contamination levels adequately is the primary reason for failures in commercial laboratories (Cassells, 2012).

Source, Vectors and Types of Microbial Contaminants Found in Plant Tissue Culture

Source

In theory, any microorganism (or its reproductive structures, e.g. spores), that is capable of growing on a plant tissue culture medium or in plant tissue *in vitro,* is potentially a contaminant. Potential microbial contaminants are those intimately associated with plant tissues and surfaces, as well as spores adhering to plant surfaces or present in the laboratory environment (Bunn and Tan, 2002). The contamination in culture medium may have an immediate or latent manifestation, in which it lies dormant for an extended period of time. Epiphytic microorganisms are microorganisms that dwell on plant surfaces and can be eradicated with chemical disinfectants, whereas the endophytes, on the other hand, invade live, interior tissues of plants without causing immediate harm and are not easily eradicated by basic surface-sterilization treatments. Resident non-pathogenic bacteria present in the initial explant are a common source of endogenous contaminants. However, superficial contaminants may be traced to residual surface microflora on explants that have survived ineffectual surface treatment or, in the case of clean established cultures, from chance introduction or cross-contamination due to poor aseptic technique. A faulty autoclave or insufficient autoclaving can be a source of contamination, but this can be identified from the consistent and even distribution of contaminant fungal and bacterial colonies within the body of the medium rather than as surface growths (Leifert and Waites, 1994).

The nutrient media in which the plant tissue is cultivated is a good source of nutrient for microbial growth. These microbes compete adversely with plant tissue culture for nutrient. The presence of these microbes in these plant cultures usually results in increased culture mortality, while the presence of latent infections can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Varghese and Joy,

2016). Tissue culture techniques involve growing stock plants using methods that minimize microbial contamination such as: treating the plant materials with sterilizing agents (chemotherapeutics), sterilizing tools used for aseptic manipulation of explants and autoclaving the vessels and media in which cultures are grown (Binetury *et al*., 2019).

Microbial contaminants

Over 30 genera of bacteria are known to be associated with plants (Suárez-Moreno *et al*., 2012). In theory, any bacterium that is associated with explant tissue as an epiphyte, endophyte or pathogen is a potential contaminant in plant tissue culture. Orlikowska *et al.* (2017) lists several different bacteria which have been isolated as contaminants in tissue and cell cultures of a wide range of plant species. Overall, Gram-positive and Gram-negative bacteria appear equally common. *Bacillus* spp. and *Pseudomonas* spp. are well represented among the Gram-positive and Gram-negative isolates, respectively. About thirty-one micro-organisms from ten different plant cultivars growing in micropropagation have been isolated, identified and characterized, with Yeasts, *Corynebacterium* spp. and *Pseudomonas* spp. being predominant (Mose, 2015). *Bacillus* sp., *Corynebacterium* sp. and an Actinomycete have also been found contaminating the *in vitro* culture of apple rootstocks (Singh, 2018). Odutayo *et al.* (2004) had also reportedly associated the following bacteria: *Pseudomonas syringae* pv *phaseolicoli*, *Bacillus licheniformis*, *Bacillus subtilis*, *Corynebacterium* sp and *Erwinia* sp with the contamination of *Hibiscus cannabinus* and *Telfaria occidentalis* in Nigeria.

For fungi, Cassells (2012) identified common fungal contaminants at the genus level to generally be air-borne or dust-borne (e.g. *Alternaria, Aspergillus, Botrytis, Candida* (yeast), *Cladosporium, Epicoccum, Microsporum, Mucor, Penicillium, Phialophora, Rhizopus, Rhodotorula* (yeast), *Trichoderma*) or associated with soil e. g. *Fusarium.*

Yeast has also been identified as one of the predominant *in vitro* culture contaminants (Mose, 2015). Cassells (2012) further reported that over 20 species of yeasts, 32 species of bacteria and 6 species of mycoplasma are found on and in the tissues of some common agricultural and or horticultural species.

Recalcitrant microbial contaminants

Disinfectants are antimicrobial products that incorporate one or more active substances such as chlorine, iodine, alcohols, hydrogen peroxide, silver, chlorhexidine, triclosan and quaternary ammonium compounds (van Dijk *et al*., 2022). These disinfectants are incorporated in the sterilization protocol of tissue culture techniques of different plants at predetermined concentrations and exposure times. Different types of microorganisms have showed varied resistance against these sterilants, depending on the active substance of each disinfectant.

In this context, bacterial and fungal contaminants that have shown resistance to the sterilization protocol of establishing plants in *in vitro* cultures can be considered as "recalcitrant". The following pathogens have been identified to show persistent resistance to hypochlorite-based disinfectants: *Staphylococcus aureus, Streptococcus*, *Enterococcus*, *Pseudomonas aeruginosa*, *Clostridium difficile*, *Salmonella*, *E. coli*, *Acinetobacter baumannii* and *Mycobacterium tuberculosis* (Mose, 2015). Odutayo *et al*. (2004) reported that these microorganisms were also isolated in Nigeria biosafety laboratories that they investigated. Generally, the order of resistance of some microorganisms against disinfectants/sterilants is shown in Figure 2.

Control methods for microbial contamination

According to Bunn and Tan (2002), different strategies are employed in plant tissue culture to contain microbial contamination. These strategies include the following:

Avoidance/in vivo strategies: These strategies are ultimately dependent on (a) selecting the type of explant material (seeds, shoot tips, nodes, stem, leaf, floral, root, bulb scales), (b) selection of the appropriate stage of development, and (c) scope for pre-treatment of the starter material *in vivo.*

In vitro Strategies: According to Waheeda and Shyam (2017), the use of general biocides in *in vitro* control strategies were highlighted in their review and they further detailed the recommended concentrations and exposure times for three chemical biocides (i.e. sodium and calcium hypochlorite, and mercuric chloride), and the species and type of explant materials treated. These sterilants are used at relatively low concentrations (typically 0.1% for mercuric chloride, 0.5% for sodium hypochlorite (NaOCl) and 3-5% for calcium hypochlorite), and ranging from a few minutes of treatment for soft shoot material up to 45 minutes for dormant buds and seeds.

Chlorine compounds should be effective in most instances but there is a limit to the concentration of NaOCl that can be used on vegetative materials before oxidative damage becomes problematic. Mercuric chloride by comparison is highly toxic at a relatively low concentration (Bunn and Tann, 2002), which makes it a very effective surface sterilant but problematic in terms of disposal due to the inherent occupational health and environmental hazards. They submitted that the accumulated experience with local flora is invaluable in the choice of appropriate sterilisation protocols for the explant material as nearly 70% of the adopted procedures resulted in a 50% success rate, which must be considered highly satisfactory for field-collected material.

Heat treatment or thermosterilisation of *in vitro* cultures is another elimination strategy usually aimed at virus elimination in excised shoots and shoot meristems. However, it has also been used to rid explants of other

microbial contaminants (Panattoni *et al*., 2013). This technique has been described to be more effective in combination with salicyclic acid (SA) and its derivative acetyl salicyclic acid (ASA) in spray form as reported by Khan *et al.* (2015). Other methods include**:**

Sonication: This is the use of ultrasound which can be applied as a pre-treatment to assist in dislodging contaminants and is generally used in conjunction with chemical sterilants (NaOCl, CaOCl or other sterilant) to give the best result (Misra and Misra, 2012).

Electrosterilisation - which involves a brief exposure of plant material to a low level of electric current and it has been used with some success for the elimination of virus from potato (Adil *et al*., 2022).

Use of chemotherapeutics in plant tissue culture

The use of chemotherapeutics such as antibiotics in plant tissue culture has been widely reported. Mbah and Wakil (2012) reported that antibiotics used in plant tissue culture should be soluble, stable, unaffected by the components or pH of the medium, lack side effects, broadly active, non-resistance inducing, inexpensive and non-toxic to humans. They further stated that attempts have been made to suppress/eliminate endogenous bacteria from plant cultures with antibiotics with varying degrees of success. Often, bacterial growth is only suppressed (bacteriostatic effect) by antimicrobial treatments and when chemicals are removed, the bacteria resume growth (Cassells, 2012). In other instances, antibiotics effective on isolated organisms cannot be used for treating contaminated plants due to phytotoxicity or poor penetration into tissues (Singh *et al*., 2020). Although phytotoxicity and development of antibiotic-resistant bacterial populations have restricted the use of antibiotics, these side effects can be taken care of by the use of combinations of antibiotics at relatively lower concentrations (Yang *et al*., 2021).

According to Ray and Ali's (2018) review, microbial contamination mitigation using antibiotics have been reported by several authors during *in vitro* propagation of different plants such as rubber (*Hevea brasiliensis* Muell. Arg), hazelnut, *Withania somnofera, Piper nigrum, Piper colubrium* and taxus *Baccata* subsp., *Wallichiana*, *Jatropha curcus*, banana, orange i.e. *Citrus sinensis* L. Osbeck cv. Madame Vinous and sweet orange i.e. *C. sinensis* cv. Valencia. contaminants. Broad spectrum antibiotics are often used in the management of bacterial contamination. Specifically, antibiotics such as tetracycline, streptomycin, vancomycin, rifampicin, gentamycin, cefotaxime are usuaĺly incorporated in culture media to inhibit the growth of these bacterial contaminants (Wakil and Mbah, 2012). These antibiotics can be used in combinations to achieve better results. A novel method that has been reported for eliminating bacterial contamination in *in-vitro* propagation of *Moss protonema* is by the agar embedding system where antibiotics were added to the agar and embedded on to protonema, thereby, reducing the microbial growth due to the continuous contact between the tissues and antibiotics (Carey *et al*., 2015). Contamination in *Gauda angustifolia* Kunth, detected as endophytes has been treated by the administration of kanamycin and streptomycin sulphate, where kanamycin at a concentration of 10µg/ml exhibited best results with no phytotoxicity (Sood *et al*., 2012). Aside from phytotoxicity and the development of resistant microbial strains, long-term antibiotic treatment of cells or tissues can cause changes in the genetic composition of organelles (the cytoplasmic genes or cytoplasmic DNA) as well as the development of resistance in bacterial cells (Abdi *et al*., 2008). Consequently, identification of contaminants (type) prior to antibiotic therapy is the first step to combating the problem as it will ensure the selection of appropriate antibiotic for therapeutic purposes (Ray and Ali, 2018) which has been reported by several authors for several plants including Hazelnut (Reed and Tansprasert,1995), *Withania somnofera, Piper nigrum, Piper colubrium* and taxus *Baccata*sub sp. *Wallichiana* (Kulkarni *et al*., 2007); *Jatropha curcus* (Misra *et al*., 2009) and bamboo (*Guadua angustifolia* Kunth) (Nahda *et al*., 2012). According to Franco-Duarte *et al.* (2019), fast detection and identification of microorganisms is a challenging and significant feature from industry to medicine. Standard approaches are known to be very time-consuming and labor-intensive (e.g. culture media and biochemical tests). Conversely, screening techniques demand a quick and low-cost grouping of bacterial/fungal isolates. Current microbial contaminant analysis, therefore, calls for broad methods to microorganism identification which should involve the application of molecular techniques such as the sequencing of 16S ribosomal RNA gene.

The commonly used fungicide in culture media is Bavistin (50% carbendazim) at a concentration range of 150-300mg/L. This fungicide when incorporated into media showed significant reduction in fungal contamination (Panathula *et al*., 2014). Bavistin has also been reported to cause shoot proliferation in *Stevia rebaudiana* cultures (Preethi *et al*., 2011). The use of other fungicides such as ProClin®300, mancozeb and thiabendazoles were reported in controlling the contamination of yeast in apple cultures (Kolozsvári *et al*., 2005).

The use of antiviral agents such as ribavirin (RBV) (virazole), azidothymidine, and 2-thiouracil against viral diseases in plant tissue culture has also been reported (Chauhan *et al*., 2019). Some antiviral agents such as inosine monophosphate dehydrogenase (IMPDH) inhibitors, S-adenosylhomocysteine hydrolase inhibitors, and neuraminidase (NA) inhibitors are generally used in plant chemotherapy (Panattoni *et al*., 2013). Chinestra *et al.* (2015) reported the use of chemotherapy to completely eliminate Lily symptomless virus prior to the application of meristem tip culture technique. Other chemotherapeutics such as Plant Preservative Mixture (PPM) has been prescribed as a very effective, phyto-safe broad spectrum

microbicide for use in plant tissue cultures (Plant Cell Technologies, 2019). The commercial formulation based on the US patent 5,750,402 is comprised of two isothiazolones, namely 2-methyl-3 (2H) isothiazolinone (MIT) and 5-chloro 2-methyl 3 (2H) isothiazolinone (CMIT) (Guri and Patel, 1998). PPM controls microbes by penetrating the microbial cell wall and inhibiting several essential enzymes in the citric acid cycle and electron transport chain (Plant Cell Technologies, 2019).

Some other essential micronutrients such as copper has been found to have antimicrobial effect when incorporated into plant tissue culture growth media at certain levels of concentrations. The broad spectrum antimicrobial efficacy of copper has been demonstrated against several species of bacteria, fungi and viruses (Grass *et al*., 2010; Gyawali *et al*., 2011), and has been reported to also have algicidal, moluscicidal and herbicidal properties (Rajmohan *et al*., 2010).

Conclusion

Due to the negative impact of microbial contamination in the plant tissue culture industry, measures to control and substantially eliminate this occurrence are deemed highly vital. The wide range of factors (environmental, source and type of explant/crop, sterilization methods, *etc*) influencing this undesirable phenomenon of contamination emphasizes the need for continuous research and optimization of existing control/elimination techniques. However, the benefit of employing any control/elimination strategy against microbial contamination in *in vitro* propagation must not outweigh its potential deleterious effect, as this may result to an outright loss of plant resources.

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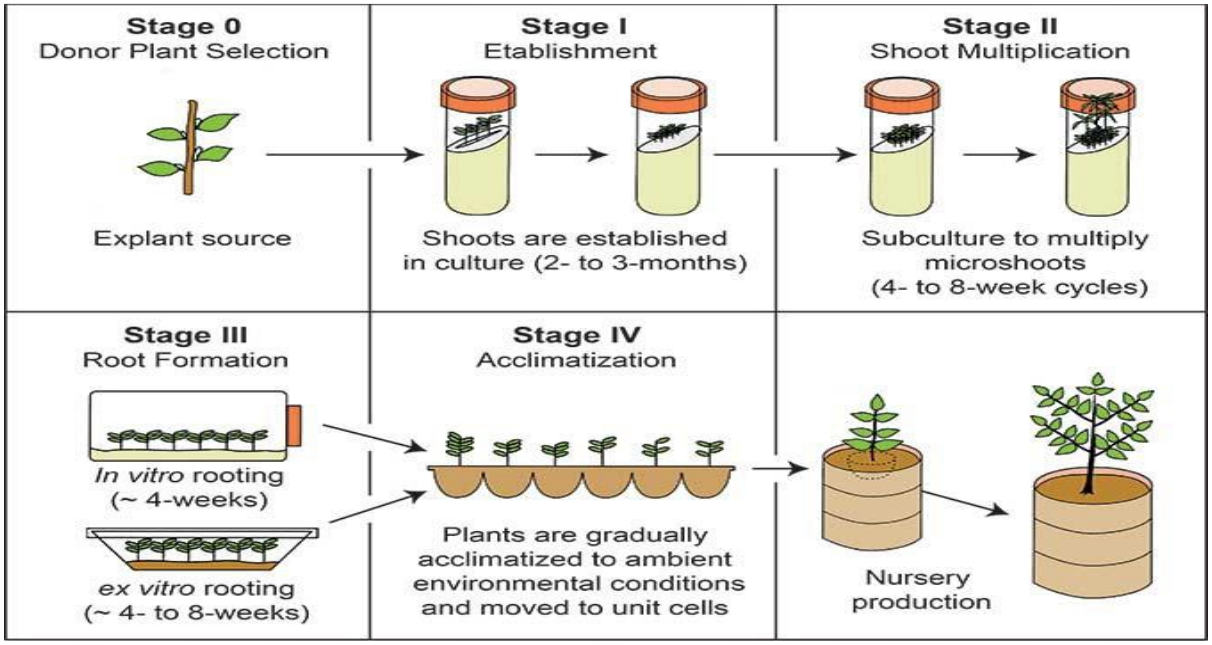

Figure 1: Different stages of micro-propagation
Source: University of Florida (2020)

Bacterial endospore (*Bacillus*, *Clostridium*)
↓
Mycobacteria (*M. tuberculosis*)
↓
Small non-enveloped viruses (*Poliovirus*)
↓
Gram-negative bacteria (*Pseudomonas*)
↓
Fungi (*Candida, Aspergillus*)
↓
Large non-enveloped viruses (*Entero*, *Adeno*)
↓
Gram-positive bacteria (*Staphylococcus*, *Enterococci*)
↓
Lipid enveloped viruses (HIV, HBV)

Figure 2: Resistance of microorganisms against disinfectant in descending order
Soyrce: McDonell and Russell (1999)

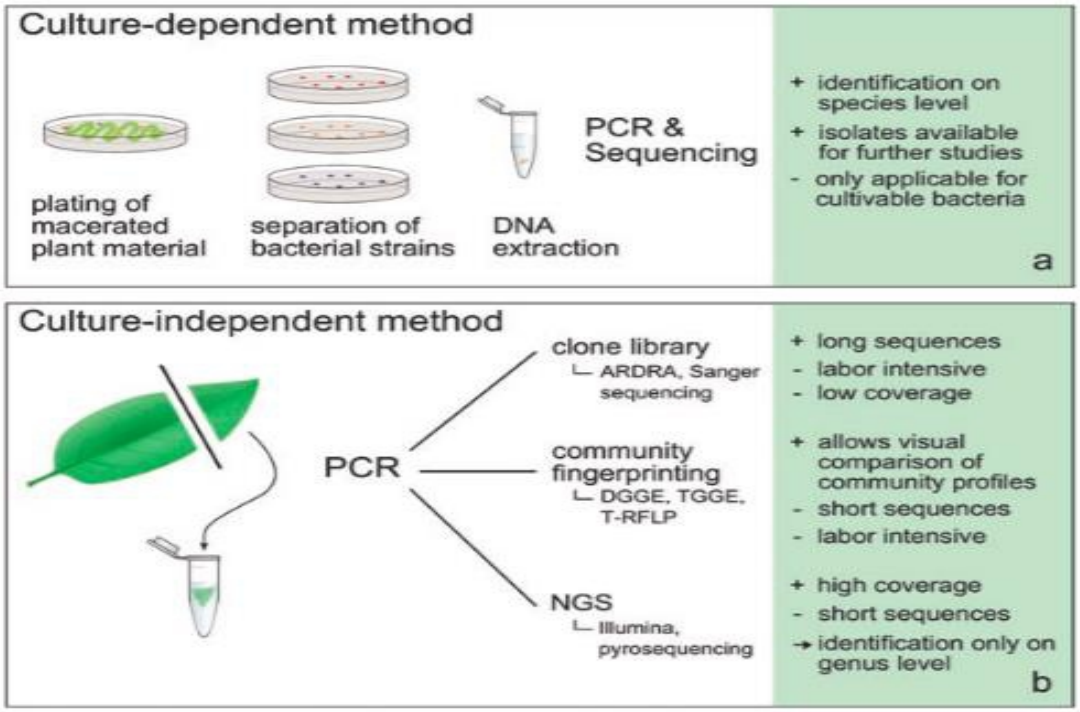

Figure 3: Different methods for microbial identification
Source: Quambusch and Winkelmann (2018)