CHAPTER 1

The Genus Pantoea in Plant Pathology; Literature Review

INTRODUCTION

The genus *Pantoea* was established in 1989 (Gavini et al., 1989) and it is classified in the family *Enterobacteriaceae*. *Pantoea* species are associated with plants as pathogens, saprophytes and epiphytes (Cottyn et al., 2001; Gavini et al., 1989; Gitaitis et al., 2000; Grimont and Grimont, 2005; Iimura and Hosono, 1996; Perombelon, 1981; Walcott et al., 2002). Strains of *P. ananatis* and *P. agglomerans* were reported to cause diseases in humans (De Beare et al., 2004; Gavini et al., 1989; Kratz et al., 2003; Lim et al., 2006; Maki et al., 1976).

Bacteria belonging to the genus (Grimont and Grimont, 2005) are gram-negative, non-capsulated, non-spore forming straight rods; most are motile and are peritrichously flagellated; are facultatively anaerobic, oxidase negative and catalase positive; colonies on nutrient agar are round, smooth, with entire margins; colonies might be yellow, cream/white or beige. There are seven described species within the genus *Pantoea* and these include: *P. agglomerans, P. ananatis, P. citrea, P. dispersa, P. punctata, P. terrea* and *P. stewartii,* containing two subspecies, *indologenes* and *stewartii* (Grimont and Grimont, 2005).

Bacterial wilt of maize was the first plant disease ever reported caused by a bacterium of the genus *Pantoea*. The disease is known as Stewart's wilt, to honour F. C. Stewart, who described the symptoms in 1897 on sweet corn in the USA (Stewart, 1897). At that time, the bacterium causing the disease was named *Bacillus stewartii.* It was also known as: *Bacterium stewartii, Erwinia stewartii, Pseudobacterium stewartii, Pseudomonas stewartii, Phytobacterium stewartii* and *Xanthomonas stewartii* (Brenner et al., 1984). The pathogen has been responsible for serious crop loses since its first discovery (Coplin et al., 2002). In 1993, the bacterium was transferred to the genus *Pantoea* as *Pantoea stewartii* subsp. *stewartii* (Mergaert et al., 1993).

Although the pink disease of pineapple was originally described in 1915 in Hawaii (Lyon et al., 1915), the pathogen responsible for this disease remained unknown until 1997. Cha et al. (1997) identified the bacterium causing the pink disease as *Pantoea citrea,* using molecular techniques. In 1928, Serrano (1928) reported brown rot of pineapple fruitlets, caused by *P. ananatis*, also in Hawaii. In the same year, Brown (1928) described a bacterial pocket disease of sugar beet, caused by *P. agglomerans*. In 1934, *P. agglomerans* was reported to induce galls on *Gypsophila* (Brown, 1934).

During the following 47 years, only one report was published, in 1954, on *P. ananatis* parasitic on uredia of cereal rust (Pon et al., 1954). Since 1981, however, bacterial species within the genus *Pantoea* have become increasingly important as plant pathogens worldwide. Seventeen new diseases caused by *P. ananatis, P. agglomerans, P. punctata* and *P. stewartii* subsp. *indologenes* have been described on a variety of crops (Table 1), including beetroot (Burr et al., 1991), cantaloupe (Bruton et al., 1991), cotton (Medrano and Bell, 2006), *Eucalyptus* (Coutinho et al., 2002), honeydew melon (Wells et al., 1987), maize (Paccola-Meirelles et al., 2001), mandarin orange (Kageyama et al., 1992), millet (Frederickson, 1997), onion (Gitaitis and Gay, 1997), pear (Lindow et al., 1998), rice (Azegami et al., 1983; Cother et al., 2004) and sudangrass (Azad et al., 2000). Disease symptoms are diverse and include galls, rots, wilt, leaf blights, necrosis and spots, dieback and stem necrosis (Grimont and Grimont, 2005). In the case of infection of honeydew melons, pineapple and cantaloupe fruit, the symptoms occur only after harvest (Bruton et al., 1986; Wells et al., 1987; Bruton et al., 1991, Cha et al., 1997).

Despite increasing frequency of plant diseases caused by *Pantoea* species, identification and characterisation of these bacteria were usually based on colony morphology, few physiological and biochemical tests and sometimes fatty acids analyses. Only *P. ananatis* strains causing dieback of *Eucalyptus* (Coutinho et al., 2002) and stem necrosis of rice (Cother et al., 2004) have been identified based on phenotypic and genotypic characteristics. Consequently, relatively little is known about isolation, identification, host specificity, genetic relatedness or epidemiology of these bacterial pathogens.

This review focuses on the detection, pathogenicity, ecology and taxonomy of the bacteria belonging to the genus *Pantoea.* The role of *Pantoea* species in plant pathology is

demonstrated and a list of *Pantoea* species causing diseases is presented. The techniques used in the diagnosis of plant diseases, and the methods for the identification of bacterial pathogens are discussed.

THE GENUS *PANTOEA*

In the past, all phytopathogenic bacteria in the family *Enterobacteriaceae* belonged to the genus *Erwinia*. The genus was proposed by Winslow et al. (1917) for all plant-associated gram-negative, non-spore forming, fermentative, rod-shaped bacteria (Beji et al., 1988). In the 1960's, Dye divided the genus *Erwinia* into four "natural" groups, founded on the type of disease symptoms produced on plants (Verdonck et al., 1987): amylovora (Dye, 1968) causing dry necrotic or wilt diseases; carotovora (Dye, 1969a) inducing soft rots; herbicola (Dye, 1969b) containing saprophytes and putative plant pathogens and atypical erwinias (Dye, 1969c).

The Herbicola group, of interest in this review, consisted of *Erwinia* strains that usually produced a yellow pigment on general growth media, including nutrient agar (Dye, 1969b; Beji et al., 1998). In 1974, Lelliot (1974) included four species in the Herbicola group. These were: *Erwinia uredovora* (Dye, 1963b), *E. milletiae* (Dye, 1969b), *E. stewartii* (Dye, 1963a) and *E. herbicola* (*E. herbicola* var. *herbicola* and *E. herbicola* var. *ananas*) (Dye, 1969b).

In 1971, Herbicola bacteria were involved in a nosocomial septicemia outbreak in the United States, when 40 of 378 patients died (Maki et al., 1976; Brenner et al., 1984). After comparing human and plant isolates, Ewing and Fife (1972) concluded that all strains belonged to a single species, which were named *Enterobacter agglomerans.* That conclusion was confirmed in 1986 by examining the DNA relatedness among 86 strains of *Enterobacter agglomerans,* isolated from humans, and the type strains of *Erwinia herbicola* and *E. milletiae* (Lind and Ursing, 1986). However, phytopathologists preferred to use the nomenclature of Dye while medical bacteriologists used the nomenclature of Edwig and Fife (Beji et al., 1988). This group of organisms was referred too in the literature as the "Erwinia herbicola-Enterobacter agglomerans complex" (Grimont and Grimont, 2005).

In 1984, Brenner et al. (1984) studied 124 strains isolated from plants and humans that belonged to the "Erwinia herbicola-Enterobacter agglomerans complex". The results of the

DNA-DNA hybridisations showed that 90 of these strains formed 13 hybridisation groups, numbered I-XIII. The authors demonstrated the immense genetic diversity of these bacteria and inadequacy of the nomenclature used at the time.

In 1987, Verdonck et al. (1987) performed the numerical analysis of phenotypic features of 529 enterobacterial strains, including those assigned to the "Erwinia herbicola-Enterobacter agglomerans complex" and other *Erwinia* species. The strains received as *Enterobacter agglomerans, Erwinia herbicola, E. milletiae, E. ananas, E. uredovora* and *E. stewartii* were dispersed into 23 phenotypic groups or phena. However, the type strains of *Enterobacter agglomerans, Erwinia herbicola and E. milletiae* grouped into a single phenon 8, the type strains of *E. ananas* and *E. uredovora* into phenon 12 and eight strains of *E. stewartii* into phenon 29.

In 1988, Beji et al. (1988) studied the DNA relatedness of the type strain *Enterobacter agglomerans*, ATCC 27155, to 54 strains from the "Erwinia herbicola-Enterobacter agglomerans complex". These 54 strains represented phenons 7B and 8 of Verdonck et al. (1987) and hybridisation groups V and XIII of Brenner et al. (1984). Seventy-three reference strains belonging to different species of the family *Enterobacteriaceae* were also included. "Erwinia herbicola-Enterobacter agglomerans complex" strains produced seven distinct protein profiles and showed 62-97% DNA binding to the *Enterobacter agglomerans* type strain, forming the hybridisation group 27155. The group included the type strains of *Enterobacter agglomerans, Erwinia herbicola* and *E. milletiae*. The authors concluded that these species names are subjective synonyms.

In 1989, based on the research of Beji et al. (1988), a new genus, *Pantoea,* was proposed (Gavini et al., 1989), with the type species, *Pantoea agglomerans*. The new species accommodated the type strains of *Enterobacter agglomerans*, *Erwinia herbicola* and *E. milletiae*, as well as the other strains belonging to electrophoretic protein profile groups I and III to VI of Beji et al. (1988) and DNA hybridisation group XIII of Brenner et al. (1984). At the same time, the second species, *P. dispersa*, was proposed, for the strains of the DNA hybridisation group III of Brenner et al. (1984) and phenon 10 of Verdonck et al (1987).

In 1992, bacterial strains were isolated from fruit and soil samples in Japan. The strains were classified as three new species within the genus *Pantoea*, namely *P. citrea, P. punctata* and

P. terrea (Kageyama at al., 1992). Grimont and Grimont (2005) suggested that additional taxonomic research was necessary to validate the placement of these three species within the genus *Pantoea*". The suggestion was based on the phylogenetic analysis of the 16S rRNA gene sequences and the *rpoB* gene sequences of all *Pantoea* species. *P. citrea, P. punctata* and *P. terrea* formed a separate cluster in phylogenetic trees that "joined the cluster containing other described *Pantoea* species at a lower level" (Grimont and Grimont, 2005).

In 1993, *Erwinia ananas* and *Erwinia uredovora* were united as a single species and transferred to the genus *Pantoea* as *P. ananatis* (Mergaert at al., 1993). *P. ananatis* comprised strains belonging to the hybridisation group VI of Brenner et al. (1984) and phenon 12 of Verdonck et al. (1987). The species *Pantoea stewartii* (Mergaert at al., 1993) contained two groups of strains. The first group, received as *Erwinia stewartii*, was originally isolated from maize or from corn flea beetles and formed phenon 29 of Verdonck et al. (1987). The DNA binding values among these strains were 93 to 99% and they were named hybridisation group 2715. The second group, DNA hybridisation group 2632, included strains received as *Erwinia herbicola* and *E. ananas* that were part of phenon 13 of Verdonck et al. (1987), and were very similar biochemically to *P. ananatis*. The DNA binding values among the strains of these two groups were between 60 and 83% and they were assigned to two subspecies of *P. stewartii*: The hybridisation group 2715 was named *P. stewartii* subsp. *stewartii* and the hybridisation group 2632, *P. stewartii* subsp. *indologenes.*

There are also four hybridisation groups from a study by Brenner et al. (1984); groups I, II, IV and V; which according to the latest edition of the Bergey's Manual of Systematic Bacteriology, belong to the genus *Pantoea* (Grimont and Grimont, 2005), but have not yet been described.

PANTOEA SPECIES AS PLANT PATHOGENS

Members of the genus *Pantoea* are mainly plant-pathogenic and plant-associated bacteria. A list of diseases caused by these pathogens is presented in Table 1.

Table 1. The diseases of plants caused by bacteria belonging to the genus *Pantoea*.

Host and Pathogen	Disease Name	References
Baby's breath (*Gypsophila paniculata*)		
P. agglomerans	Galls	Brown, 1934
		Cooksey, 1986
Beetroot (*Beta vulgaris*)		
P. agglomerans	Root galls	Brown, 1928
		Burr et al., 1991
Cotton (*Gossypium hirsutum*)		
P. agglomerans	Seed and boll rot	Medrano & Bell, 2006
Eucalyptus		
P. ananatis	Bacterial blight and dieback	Coutinho et al., 2002
Foxtail millet (*Setaria italica*)		
P. stewartii subsp. indologenes	Leaf spot	Mergaert et al., 1993
Maize (*Zea mays*)		
P. stewartii subsp. *stewartii*	Stewart's wilt	Stewart, 1897
P. ananatis	Leaf spot	Paccola-Meirelles et al., 2001
Mandarin orange (*Citrus nobilis*)		
P. citrea	Pink disease	Kageyama et al., 1992
P. punctata	Brown spot	Kageyama et al., 1992
Melon and cantaloupe (*Cucumis melo*)		
P. ananatis	Soft rot	Bruton et al., 1986
		Wells et al., 1989
	Postharvest rot	Bruton et al., 1991
Onion (*Allium cepa*)		
P. agglomerans	Leaf and seed stalk necrosis	Hattingh & Walters, 1981
	Leaf blight and bulb rot	Edens et al., 2006
P. ananatis	Leaf blight, seed stalk rot, and bulb decay,	Gitaitis & Gay, 1997
		Schwartz & Otto, 2000

Host and Pathogen	Disease Name	References
	Center rot	Walcott et al., 2002
Pear (*Pyrus communis*)		
P. agglomerans	Russetting of pear fruits	Lindow et al., 1998
Pearl millet (*Penissetum glaucum*, *P americanum*)		
P. agglomerans	Necrosis of the leaf tips and margins	Frederickson, 1997
P. stewartii subsp. indologenes	Leaf spot	Mergaert et al., 1993
Pineapple (*Ananas cosmosus*)		
P. ananatis	Bacterial fruitlet brown rot	Serrano, 1928
P. citrea	Pink disease	Lyon, 1915
		Kageyama et al., 1992
		Cha et al., 1997
Rice (*Oryza sativa*)		
P. ananatis	Palea browning	Azegami et al., 1983
	Stem necrosis	Cother et al., 2004
Rust on cereals		
P. ananatis	Parasite on uredia	Pon et al., 1954
Sudangrass (*Sorghum sudanense*)		
P. ananatis & *P. stewartii* subsp. *indologenes*	Leaf blotch	Azad et al., 2000
Sugarcane (*Saccharum* spp.)		
P. ananatis	Soft rot	Serrano, 1928

Many plant pathogenic *Pantoea* species are seed-borne and seed-transmitted, such as *P. ananatis* in onion (Walcott et al., 2002), sudangrass (Azad et al., 2000), rice (Tabei et al., 1988), buckwheat (Iimura and Hosono, 1996) and *P. agglomerans* in cotton (Medrano and Bell, 2006). *P. stewartii* subsp. *stewartii*, the causal agent of Stewart's wilt of maize, is also seed-borne and many countries have banned the importation of maize seed, unless they are certified free of *P. stewartii* (Coplin et al, 2002).

Pantoea stewartii subsp. *stewartii* infects its host plant systemically. It multiplies quickly and produces exopolysaccharide materials in the vascular system. Wilting of maize plants is caused by the blockage of vessels by exopolysaccharides (Grimont and Grimont, 2005; Pepper, 1967). The corn flea beetle (*Chaetocnema publicaria*) is a host of *P. stewartii* subsp. *stewartii* during winter. Throughout the growing season, the pathogen is spread from plant to plant and from field to field by the beetle. The epidemics of Stewart's wilt usually occur after mild winters that facilitate the survival of the insect vector (Grimont and Grimont, 2005; OEPP/EPPO, 1978). *P. ananatis,* causing center rot of onion, is transmitted via tobacco thrips, *Frankliniella fusca* (Gitaitis *et al.*, 2003; Wells *et al.*, 2002*). P. ananatis* has also been reported as a gut inhabitant of brown planthoppers, *Nilaparvata lugens* (Watanabe et al., 1996) and mulberry pyralids, *Glyphodes pyloalis* (Watanabe and Sato, 1999), but none of these insects are a vector of the pathogen.

The tumorigenic *P. agglomerans*, which induces galls on *Gypsophila* and beetroot and *P. citrea* causing pink disease of pineapple, are spread mechanically and through infected plant propagation material (Cooksey, 1986; Burr et al., 1991; Cha et al., 1997). *P. agglomerans* strains that produce plant hormones may also induce diseases. For example, *P. agglomerans* strains producing indole acetic acid and cytokinin (Guo et al., 2001) cause galls formation on *Gypsophila* (Manulis et al., 1998) and russeting of pear fruits (Lindow et al., 1998). Some strains of indoleacetic acid-producing *P. agglomerans* increase the severity of the olive knot disease, caused by *Pseudomonas savastanoi* pv. *savastanoi* (Marchi et al., 2006).

Some *P. agglomerans* and *P. ananatis* strains are ice nucleation active (Hirano and Upper, 2000). The ice nucleation active bacteria, when present on plant surfaces, increase the chances of frost injury at subzero temperatures (Hirano and Upper, 1995; Hirano and Upper, 2000; Lindow et al., 1982). *P. agglomerans*, found as an epiphyte on leaves of maize, was reported to enhance the frost injury in this host (Lindow et al., 1978). The ice nucleation active *P. ananatis* was isolated from the gemmisphere of tea, phyllosphere of many vegetables, the flowers of magnolia (Goto et al., 1988; Goto et al., 1993) as well as from wheat and barley (Newton and Hayward, 1986).

PANTOEA SPECIES AS BENEFICIAL MICROORGANISMS IN AGRICULTURE

Not all *Pantoea* strains are plant pathogens. Many strains have been used for biological control of other plant pathogens. In general, "an effective biological control agent has to be able to live and multiply in the same ecological niche as the pathogen" (Özactan and Bora, 2004; Pusey, 1997; Vanneste et al., 1999). There are two main mechanisms, by which biological control strains hamper the growth of pathogens.

The first mechanism is competition for sites, growth space and growth-limiting nutrients. *P. agglomerans* strains were reported to suppress the fire blight pathogen, *Erwinia amylovora*, in pear fruits and apple blossoms (Beer et al., 1984; Özactan and Bora, 2004). Application of naturally occurring Ice$^-$ strains of, among others, *P. agglomerans*, significantly reduced frost injury to sensitive crops by (Hirano and Upper, 2000; Lindow, 1982; Lindow et al., 1983; Wilson and Lindow, 1994). The post-harvest control of blue mould on pome fruits, caused by *Penicillum expansum* was achieved by application of epiphytic *P. agglomerans* and *P. ananatis* isolated from the fruits and leaf surfaces of apples and pears (Nunes et al., 2001; Torres et al., 2005).

The second biocontrol mechanism is production of bio-fungicides and bio-bactericides that inhibit the growth of pathogens. A strain of *P. dispersa* producing chitinase has been used as a biocontrol agent against fungal plant pathogens (Gohel et al., 2004). A strain of *P. agglomerans* is effective in controlling many banana pathogens (Gunasinghe et al., 2004). *P. agglomerans* strains control damping-off caused by *Phythium* species on canola, safflower, dry pea and sugar beet (Barding et al., 2003). A strain of a *Pantoea* species was reported to induce resistance in cucumber against *Pseudomonas syringae* pv. *lachrymans*, the agent of angular leaf spot (Hoon et al., 2006). A strain of *P. citrea* that contains the gene for albicdin detoxification has been reported to attenuate the pathogenicity of *Xanthomonas albilineans* to sugarcane (Zhang and Birch, 1997a). The gene is a promising candidate to transfer into sugarcane to confer a form of resistance against leaf scald (Zhang and Birch, 1997b).

Many researchers isolated and identified free-leaving nitrogen-fixing bacteria from leaves and stems of diverse plants (Hirano and Upper, 2000; Ladha et al., 1983; Ruinen, 1975; Sprent and Sprent, 1990). In 2004, a nitrogen-fixing *Pantoea* sp. was isolated from sugarcane in Cuba that may be valuable in agriculture (Loiret et al., 2004). The strain of *P.*

agglomerans, isolated from the rhizosphere of wheat in Morocco, had a positive effect on the aggregation and macroporosity of rhizosphere soil. The ultimate goal of the researchers is to use that exopolysaccharide-producing bacterium as inoculant in wheat fields to regulate water stress and, therefore, improve yields (Amellal et al., 1998).

ISOLATION OF *PANTOEA* SPECIES FROM PLANTS

Plant disease diagnosis and the identification of pathogen consist of several steps and these usually include: isolation of a pathogen from diseased plant tissues; obtaining a pure culture of a suspected pathogen; microscopic examination, for example to determine the Gram-stain reaction; serology, if a pathogen-specific antibodies are available; phenotypic tests including nutritional, physiological and biochemical characterisation and DNA-based identification (Alvarez, 2004; Goszczynska et al., 2000; Houpikian and Raoult, 2002).

Alvarez (2004) and Houpikian and Raoult (2002) wrote that although DNA-based techniques have become indispensable in the detection and identification of bacterial pathogens, they could not completely replace other, more traditional methods. For example, isolation of bacteria on agar media is crucial in plant pathology. A pure culture of a pathogen allows studying both known and emerging bacterial diseases. Purified bacterial strains can be characterised in detail and easily compared with similar strains isolated by other researchers. Isolation and obtaining a pure culture of bacteria remains "the ultimate goal of pathogen identification" (Houpikian and Raoult, 2002).

Majority of plant pathogenic bacteria, including *Pantoea* species, can be easily isolated from diseased tissues, especially from freshly collected samples (Coplin et al., 2002; Goszczynska et al., 2000). When a new disease is observed, isolations should be done from many plants, to make sure that the particular bacterium is associated with a disease (Alvarez, 2004). Azad et al. (2000), for instance, used 151 affected plants in their study while investigating a new leaf blotch disease of sudangrass, caused by two *Pantoea* species.

Media specifically selective for *Pantoea* strains are not yet available, but all media designed for the isolation *of Enterobacteriaceae* can be used for the isolation of *Pantoea* species and these include: MacConkey agar, Drigalski lactose agar, blood agar and Luria-Bertani (LB) agar (Grimont and Grimont, 2005). Although LB has been the most popular agar medium for

isolation of human pathogens, plant pathologists preferred nutrient agar (NA). Most of the plant pathogenic *Pantoea* species, in the first reports, were isolated on NA, including *P. agglomerans* from onion (Hattingh and Walters, 1981) and *P. ananatis* from *Eucalyptus* (Coutinho et al., 2002).

Isolation of *P. ananatis* from onion seed on NA is difficult due to abundant growth of saprophytes. Walcott et al. (2002) tried to solve this problem by using a polyclonal antiserum against *P. ananatis*. Seed extracts were incubated with immunomagnetic beads coated with the antiserum to immuno-bind the pathogen. Then the beads with bound *P. ananatis* were plated on NA. However, 64% of colonies recovered from seed lots by immuno-plating were not *P. ananatis*.

DETERMINATION OF PATHOGENICITY

Determination of pathogenicity and fulfilment of Koch's postulates is a crucial step in the identification of plant pathogenic bacteria. Pathogenicity tests are not standardised and are dependant on the host-pathogen combination (Goszczynska et al., 2000). Many authors used several different inoculation methods to confirm pathogenicity of *Pantoea* species associated with disease symptoms.

Coutinho et al. (2002) employed two inoculation techniques for *P. ananatis* causing bacterial blight and dieback of *Eucalyptus*. Three-month-old *Eucalyptus* plants were used in pathogenicity tests. A fine needle was dipped into a bacterial suspension and then gently inserted into the surface of young leaves and into the petioles of young leaves. All plants were incubated at temperatures between 20 and 23°C and relative humidity between 80 and 90%.

Paccola-Meirelles et al. (2001) investigating leaf spot of maize, also used two inoculation methods, but tests were performed on 15-, 30-, and 45-day old plants. In the first method, a bacterial suspension was sprayed onto leaves after carefully wounding them with carborundum or puncturing them with a sterile needle. In the second method, the bacterial suspension was injected into leaves with a syringe and 26-gauge needle.

Azad et al. (2000) inoculated sudangrass using five methods: suspensions were sprayed onto leaf surfaces, infiltrated into leaf tissues with a syringe, injected into stems, mixed with an abrasive material and applied to leaves with a cloth and bacterial colonies were applied directly to wounds made in leaves and stems. Three plant growth conditions were tested for each inoculation method, which varied in temperature and relative humidity. All inoculation methods and growth conditions were favourable for development of symptoms. The infection was most rapid, however, when plants were inoculated by infiltration of bacterial suspensions into the tissues, and by direct application of bacterial colonies to needle induced wounds. Symptoms appeared 2-3 days after inoculation on such plants when they were kept in a growth chamber with a constant temperature of 32°C. In contrast, in a greenhouse with a lower temperature (20°C), symptoms were not visible until 17 days after inoculation. Cother et al. (2004) reported that stem necrosis developed in rice following inoculation of inflorescences and stems. No lesions were present on spray-inoculated leaves.

PHENOTYPIC IDENTIFICATION OF *PANTOEA* SPECIES

If a bacterium induces disease symptoms in pathogenicity tests, computerised commercial identification systems can be employed for identification. Examples include: metabolic tests, API 20E and API 50CHE (BioMérieux, La Balme les Grottes, Montalieu Vercieu, France); substrate utilisation, Biolog (Biolog, Inc., Hayward, CA) and Pheno 100 (BioMérieux) and analysis of fatty methyl esters (MIDI, Newark, DE). The obtained results are compared to that in the built-in database, and the name of the species name is given by the programme. Alvarez (2004) cautioned that the phenotypic identification systems are not always correct, as not all bacterial species are included in the databases. This is particularly true in the case of *Pantoea* species.

The phenotypic characteristics of named species and unnamed hybridisation groups of *Pantoea* are summarised in the latest edition of the Bergey's Manual of Systematic Bacteriology (Grimont and Grimont, 2005). The differentiation between *Pantoea* species based solely on phenotypic characteristics is sometimes difficult. For example, *P. agglomerans* and *P. dispersa* could be distinguished by only two tests, the ability to utilise malonate and erythitol (Gavini et al, 1989). *P. ananatis* and *P. stewartii* subsp. *indologenes*, two indole producing species of the genus, are only differentiated by the ability to produce acid from sorbitol and α-methyl-D-mannoside (Mergaert et al., 1993).

Tumorigenic *P. agglomerans* from *Gypsophila* and table beet (Burr et al., 1991; Cooksey et al., 1986), *P. ananatis* associated with a leaf spot disease of maize (Paccola-Meirelles et al., 2001), *P. ananatis* and *P. stewartii* causing leaf blotch on sudangrass (Azad et al., 2000) and *P. agglomerans* isolated from pearl millet (Frederickson, 1997), were identified solely by selected biochemical and physiological characteristics. Walcott et al. (2002) confirmed *P. ananatis* isolated from onion seed by the Hugh-Leifson assay, indole test and fatty acid methyl ester analysis.

DETECTION OF PLANT PATHOGENIC BACTERIA USING THE POLYMERASE CHAIN REACTION (PCR)

The first report about the polymerase chain reaction (PCR), titled "Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia" was published by Saiki et al. (1985). Two years later, Mullis and Falona (1987) published the paper "Specific synthesis of DNA in vitro via a polymerase chain reaction". In 1988, Saiki et al. (1988) reported about the "Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase" allowing them to make unlimited copies of a fragment of DNA. The PCR technique revolutionised research in wide-ranging fields of the biological sciences (Babalola, 2003). The impact of PCR on biology could be compared with the unravelling the structure of DNA and decoding of the genetic code. In 1995, K. Mullis received the Nobel Price in chemistry for the discovery of PCR.

In the field of phytobacteriology, numerous research groups have reported the development of pathogen specific primers, allowing detection and identification of these pathogens in heterogeneous mixtures (Alvarez, 2004). Several primers pairs were developed for the specific detection and identification of *P. stewartii subsp. stewartii* in plant material (Blakemore et al., 1992; Blakemore et al., 1999; Coplin et al., 2002). The pathogen was easily detected in the tissue extracts from infected plants. The minimum number of *P. stewartii* cells needed for the detection was 200 CFU (Coplin et al., 2002).

The PCR could be inhibited by many compounds present in the plant and seed extracts (Alvarez, 2004; Schaad et al., 1995). Immunocapture or immunomagnetic separation (IMS) has been used to overcome this problem. The role of the antibody in the IMS-PCR is to

capture the pathogen from a plant extract containing mixture of bacteria and PCR-inhibitory substances. The IMS-PCR assay developed to detect *P. ananatis* in onion seed, demonstrated detection and recovery of 10^1 to 10^4 CFU/ml of spiked seed wash (Walcott et al., 2002). Schaad et al. (1995) developed the "Bio-PCR" to enhance the sensitivity of PCR reaction. Bio-PCR detects living cells of pathogens, those that could cause a disease, as bacterial colonies are picked up from agar plates preceding the PCR reaction. A plant or seed extract is plated on semi-selective media, plates are incubated for two days (longer incubation time is required for the slow-growing bacteria) and then the bacterial growth is removed from the agar plates and suspended in sterile distilled water. This bacterial suspension is used as a template in the PCR with primers targeting specific pathogen.

In the early 1990's, Higuchi et al. (1992) demonstrated the simultaneous amplification and detection of specific DNA sequences. The technique, named the "real-time PCR" (Higuchi et al., 1993), consists of "the amplification of DNA by PCR that is monitored using fluorescent technology, while the amplification is occurring" (Valasek and Repa, 2005). This method is: extremely sensitive; detects less than five copies of a target sequence; very quick, results are obtained within 30-50 min; performed in a close reaction environment so the chances for cross contaminations are minimised (Valasek and Repa, 2005). Many researchers pointed out that recent advances in the molecular-based diagnostic techniques, especially the real-time PCR, are invaluable in early and rapid detection of pathogens in propagation material, seed and diseased crops. Correct phytosanitary measures can be implemented at once, contributing to the crop biosecurity (Martin et al., 2000; Schaad et al., 2003; Strange and Scott, 2005).

DNA FINGERPRINTING-BASED METHODS USED FOR IDENTIFICATION AND TAXONOMY OF BACTERIA

The DNA-based, fingerprinting methods used for identification and taxonomy of bacteria can be divided into three groups. The first group is the restriction fragment analysis based on the digestion of the whole genome DNA with restriction enzymes. Examples include restriction fragment length polymorphisms (RFLP) and pulsed-field gel electrophoresis (PFGE). PCR-based typing techniques comprise the second group of fingerprinting methods with random amplified polymorphic DNA (RAPD) and repetitive sequence based PCR (Rep-PCR). Amplified fragment length polymorphism (AFLP) combines the PCR and the

digestion of DNA with restriction enzymes. All these techniques have been used by countless research groups for identification, differentiation and classification of almost every cultivable bacterial group.

The perfect genomic fingerprinting technique should be applicable to all bacterial strains to be studied, reproducible, highly discriminatory, easy to do and fast (Olive and Bean, 1999; Vandamme et al., 1996). Genomic fingerprints obtained by using these techniques are usually complex and computerised analysis is necessary to interpret the results and to compare a large number of strains (de Bruijn et al., 1996; Gürtler and Mayall, 2001; Olive and Bean, 1999; Rademaker and de Bruijn, 1997; Savelkoul et al., 1999). One of the best software available for such analysis is BioNumerics (Applied Maths, Kortrijk, Belgium). None of the fingerprinting methods is perfect, of course, but some are more suitable for specific bacterial taxa than others are.

Restriction fragment length polymorphism (RFLP). The restriction fragment analysis consists of four stages: extraction of the whole-genome DNA, digestion of DNA with restriction enzyme or enzymes, agarose gel electrophoresis of DNA fragments and interpretation of results (Vandamme et al., 1996). The selection of restriction enzymes and the digestion conditions has to be determined experimentally for the group of bacteria to be studied. Additionally, the obtained patterns of DNA fragments are usually complicated, difficult to compare and not always reliable (Vandamme et al., 1996). Despite these obvious drawbacks, RFLP was used successfully in 1989 in Australia, for identification of *Ralstonia solanacearum* race 2, the causal agent of Moko disease of banana (Hayward, 1996; Hyde et al., 1992).

Pulsed-field gel electrophoresis (PFGE) also known as the low-frequency restriction fragment analysis. PFGE is regarded as the most discriminatory fingerprinting method available (Struelens et al., 2001). The PFGE technique is an improved RFLP. There are many differences, contributing to the enhancement in reproducibility, reliability and discrimination power (Olive and Bean, 1999; Trenover et al., 1995; Vandamme et al., 1996). The DNA is not extracted from the cells by conventional techniques. Bacterial strains are cultivated on an appropriate agar medium or in a broth and mixed with molten agarose to form “agarose plugs”. Bacterial cells in agarose plugs are lysed and digested with restriction enzymes *in situ.* The restriction enzymes are cutting DNA infrequently (recognise specific

combination of six to eight bases). Consequently, the number of obtained DNA fragments is smaller than in conventional RFLP, but they are large (10 to 800 kb). Special electrophoretic techniques are used to separate large DNA fragments, known as pulsed-field gel electrophoresis (PFGE).

The PFGE procedure is simple to perform and the results are reproducible, but it is time consuming and therefore, not convenient for assessment of the large number of strains (Olive and Bean, 1999). Despite the time factor the PFGE remains the "gold standard" of DNA-based typing (Olive and Bean, 1999). Gürtler and Mayall (2001) were concerned about growing evidence regarding the negative effect of mobile genetic elements on the determination of bacterial relatedness by PFGE. Plasmids and transposons present in some strains, but absent in others of the same species, may contain restriction sites for infrequent cutting enzymes, for example, up to seven *SmaI* sites (Thal et al., 1997). PFGE patterns of such strains were different, but still the strains belonged to a single species.

PFGE was evaluated to confirm the identity of *P. stewartii* subsp. *stewartii* (Coplin et al., 2002). After DNA digestion with *Spe*I and *Xba*I restriction enzymes, *P. stewartii* strains could be easily distinguished from related *Erwinia* and *Pantoea* species and from each other. PFGE analysis of *P. stewartii* revealed many common bands among strains from different geographic regions. The genome of *P. stewartii* strains was highly conserved (similarity from 60 to 100%) based on these common fragments. On the other hand, there was sufficient divergence in the PFGE profiles to differentiate between *P. stewartii* strains from different geographical regions, showing that the technique also may be a useful tool for population genetics and epidemiological studies.

Random amplified polymorphic DNA (RAPD) assay. The RAPD fingerprinting is PCR-based. The technique was named by its developers as an arbitrary primed PCR (Welsh and McCleland, 1990; Williams et al., 1990). In RAPD, short (about 10 bases long), random primers are used to produce genomic fingerprints of bacterial strains. The primers that generate the best pattern for identification or discrimination of the studied bacteria have to be selected empirically (Olive and Bean, 1999). Trebaol et al. (2001) used 340 RAPD primers to investigate the genetic diversity in *Xanthomonas cynarae*, causing bacterial bract spot of artichoke. Among these 340 primers tested, only 40 produced reproducible and reliable fingerprints.

As the RAPD primers are arbitrary (not complimentary to any specific locus on the genome), the technique is very sensitive to changes in the reaction conditions, including the annealing temperature and reagents. That sensitivity makes RAPD banding patterns difficult to reproduce (Meunier et al., 1993; Olive and Bean, 1999; Welsh and McClelland, 1990). The RAPD technique is relatively easy to execute, quick and does not require expensive laboratory equipment. Any laboratory owning a thermocycler and an electrophoresis apparatus can do it. Hence, many studies contributed to standardisation of the procedure.

Vogel et al. (1999) performed RAPD typing of *Klebsiella pneumoniae*, *K. oxytoca, Serratia marcescens* and *Pseudomonas aeruginosa* isolates using standardised reagents. The use of Ready-To-Go RAPD Analysis beads resulted in reproducible and stable banding patterns with a high discriminatory capacity. A sequencing primer, M13 used in RAPD fingerprinting assays also allows for standardisation of the procedure (Olive and Bean, 1999; Vila et al., 1996). In 2005, Rosetti and Giraffa (2005) reported that the strains of dairy lactic acid bacteria are rapidly identified by M13-generated, RAPD-PCR databases.

A web-based database for the provisional identification of bacterial species using only genotypes was developed in Japan (Watanabe et al., 2002). The PCR reactions with DNA of all kinds of bacteria are performed using a set of four RAPD primers so all species can be compared on the same platform. The PCR products are separated, using a temperature-gradient gel electrophoresis and the images are processed to generate species-identification dots, named spiddos. The protocol was standardised to make the system reproducible and reliable.

Repetitive sequence based PCR (Rep-PCR). The rep-PCR genomic fingerprints are produced by performing PCR with primers complimentary to repetitive DNA elements present within bacterial genomes. The PCR amplifies genomic regions located between the repetitive sequences, generating reproducible patterns characteristics for examined bacterial strains (de Bruijn et al., 1996; Versalovic et al., 1991). There are several families of repetitive sequences (de Bruijn, 1992; Versalovic et al., 1991; Versalovic et al., 1994), but three of them have been used extensively to characterise diverse bacterial species. These are (de Bruijn et al., 1996; Olive and Bean, 1999): the 35-40 bp repetitive extragenic palindromic (REP) sequence (Stern et al., 1984); the 124-127 bp enterobacterial repetitive

intergenic consensus (ERIC) sequence (Hulton et al., 1991) and the 154 bp BOX element (Koeuth et al., 1995).

The discriminatory power of rep-PCR increases when strains are analysed with multiple sets of primers (Olive and Bean, 1999; Rademaker et al., 2000). Rep-PCR has only slightly less discriminatory power than PFGE (Barbier et al., 1996; Georghiou et al., 1995; Liu and Wu, 1997; Weigel et al., 2004), but unlike PFGE, is simple, quick and inexpensive. Olive and Bean (1999) wrote that rep-PCR "is becoming the most widely used method of DNA typing". Purification of DNA from bacterial cells prior to rep-PCR is not essential. Many researchers obtained identical patterns using broth cultures, single colonies, extract from lesions and purified DNA as templates for the reaction (de Bruijn et al., 1996).

Rep-PCR has been used to differentiate and identify, among many others, strains of *Escherichia coli* from water (dos Anjos Borges et al., 2003), soft-rot *Erwinia* from ornamental plants (Norman et al., 2003), *Serratia marcencens* causing the cucurbit yellow wine disease (Zhang et al., 2003) and *Rhizobium meliloti* (de Bruiin, 1992). A database of rep-PCR patterns of a large collection of *Xanthomonas* isolates coupled to a computer assisted phylogenetic analysis, has been used as a tool for strain diagnosis (de Bruijn et al., 1996; Rademaker and de Bruijn, 1997).

Amplified fragment length polymorphism (AFLP). In 1993, Zabeau and Vos, (1993), patented a new technique for DNA fingerprinting of plant genomes, the selective restriction fragment amplification. Vos et al. (1995) described the method in details and named it the amplified fragment length polymorphism (AFLP). AFLP was adapted and optimised for the analysis of bacterial genomes and for the identification of bacteria by Janssen et al. (1996).

The AFLP protocol is quite complicated and contains several stages (Janssen et al., 1996; Kassama et al., 2002; Olive and Bean, 1999; Savelkoul et al., 1999; Vandamme et al., 1996). Extraction and purification of DNA from pure bacterial cultures is essential, because the AFLP fingerprints produced by the direct use of DNA from e.g. soil are not instructive. The DNA (about 100 ng) is digested with two restriction enzymes to produce DNA fragments with two types of "sticky ends", corresponding to the restriction enzymes used. One enzyme is a frequent cutter, for example *Mse*I, recognising a 4 bp restriction site. The second enzyme recognises a 6 bp site (not so frequent cutter), *Eco*RI being the most popular. Short

nucleotides (adapters) are then ligated to the restriction fragments. These adapters contain a restriction site complementary to a "sticky end" and a sequence homologous to a primer for a subsequent PCR. Pre-amplification PCR is performed with primers complimentary to the sequences of an adapter and a restriction site. Pre-amplification PCR amplifies only restriction fragments that have an adapter ligated to both ends. Selective PCR amplification is done with selective primers that are complementary to the restriction sites and have one to three selective nucleotides at their 3'-ends. A selective primer complimentary to the *Mse*I with two selective nucleotides GG will amplify only the *Mse*I sites flanked by the CC nucleotides, thus reducing the complexity of DNA fingerprints. One of the selective primers, containing the normal frequency restriction site (*Eco*RI for example) is labelled with a fluorescent dye, to visualise obtained fingerprints on gels. The PCR products are separated by a polyacrylamide gel electrophoresis and visualised by using a laser light.

The discriminatory power of AFLP is equal to that of PFGE for most bacterial taxa (Jureen et al., 2004; Keto-Timonen et al., 2003; Lindstedt et al., 2000, Olive and Bean, 1999). Keto-Timonen et al. (2003) reported that AFLP was faster than and not as laborious as PFGE when applied to the discrimination of *Listeria* isolates. In some cases, for example in differentiation of *Streptococcus pyogenes* strains (Desai et al., 1998), and recognition of individual strains of *Xanthomonas axonopodis* (Janssen et al., 1996), AFLP was superior to PFGE (Savelkoul et al., 1999).

Countless researchers have used AFLP analysis in bacterial studies. The technique was applied to the identification and taxonomy of the soft rot bacteria *Erwinia carotovora* (syn. *Pectobacterium carotovorum* subsp. *carotovorum*) and *E. chrysanthemi* (syn. *Dickeya* spp) (Avrova et al., 2002). AFLP analysis of *Klebsiella pneumoniae*, K. *oxytoca* and other *Klebsiella* species allowed the discrimination of the species within the genus and recognition of epidemiologically non-related isolates (Jonas et al., 2004). Kassama et al. (2002) used AFLP for identification of bacterial isolates from urinary tract infections. The relatedness of 69 bacteria was determined by cluster analysis of the AFLP-generated fingerprints. The bacteria grouped into eight clusters, corresponding to eight bacterial species. Brady (2005) developed an AFLP-based typing system for the genus *Pantoea*. Seventy-nine strains included in the study, both reference strains of *Pantoea* species and suspected *Pantoea* from *Eucalyptus*, formed 15 distinct clusters in the dendrogram derived from the fingerprint data.

As the genus *Pantoea* contains seven species, the results indicated that some strains could belong to new, yet undescribed species.

AFLP and rep-PCR genomic fingerprinting techniques were compared to DNA-DNA hybridisation studies (Rademaker et al., 2000). AFLP and rep-PCR genomic fingerprints (rep-PCR with three different primers sets) were produced for 178 *Xanthomonas* strains, belonging to 20 defined DNA-DNA homology groups within *Xanthomonas*. The authors calculated similarity values obtained from rep-PCR and AFLP generated fingerprints and compared them with the DNA-DNA homology values. The similarity values were highly correlated, suggesting that genomic fingerprinting using AFLP and rep-PCR shows real genotypic and phylogenetic relationships among *Xanthomonas*. The study gave evidence that rep-PCR and AFLP could be used for identification of bacterial strains, studying the taxonomic diversity of bacterial groups and determination of phylogenetic structure of bacterial populations (Rademaker et al., 2000).

Other DNA-based typing methods. Apart from RFLP, PFGE, RAPD, rep-PCR and AFLP, the introduction of molecular biological techniques into the microbiology laboratory yielded a large variety of other DNA-based typing methods. Restriction fragment analysis of plasmids, PCR-based locus specific RFLP, ribotyping, cleavase fragment length polymorphism have all been used and evaluated as tools for taxonomic investigations (Gürter and Mayall, 2001; Houpikian and Raoult, 2004; Louws et al., 1999; Olive and Bean, 1999; Rademaker et al., 2000; Vandamme et al., 1996; Versalovic and Lupski 2002).

16S rRNA GENE SEQUENCE ANALYSIS

In the 1970's, Sanger, Gilbert and Maxam developed a technique for DNA sequencing (Broughton, 2003). Today, almost 40 years later, it is difficult to imagine biological sciences without DNA sequences. There are over 400 000 sequences of the 16S rRNA gene available in the GenBank, the largest database of nucleotide sequences (National Center for Biotechnology Information, U.S. National Institute of Health, Bethesda, MD). Since the landmark studies by Woese in 1970's (Woese et al., 1975) and 1980's (Woese et al., 1985; Woese, 1987), the 16S rRNA gene sequence has become an essential tool for bacterial classification and identification (Clarridge, 2004; Vandamme et al., 1996). Clarridge (2004)

compared the impact of the 16S rRNA gene sequence on bacterial taxonomy with that of the Gram-stain, developed in 1884 by C. Gram (Broughton, 2003).

The 16S rRNA gene supports protein synthesis in bacteria, a fundamental element of every cell function (Noller et al., 2005). The 16S rRNA gene (Clarridge, 2004) is present in all bacteria, the sequence is approximately 1,550 bp long, is the most conserved bacterial gene, contains both conserved and variable regions, variable regions are used for the comparative taxonomy and is easily amplified by PCR using universal primers (Weisburg et al., 1991). The gene could be amplified directly from diseased tissue, allowing characterisation and identification of bacteria that are difficult or impossible to culture (Avilla et al., 1998; Houpikian and Raoult, 2002; Relman, 1999)

Kwon et al. (1997) and Hauben et al. (1998) showed, using 16S rDNA sequences of plant associated strains, representing *Erwinia, Pantoea* and other *Enterobacteriaceae*, that three phylogenetic groups exist within *Pantoea*. *Pantoea* species group in a monophyletic unit that is closely related to the true *Erwinia* species. The results indicated that 16S rDNA sequences could be used to differentiate and identify bacteria of the family *Enterobacteriaceae*, including the genus *Pantoea*.

The 16S rDNA sequences have been used, together with phenotypic characterisation, in identification of *P. ananatis* causing stem necrosis of rice (Azad et al., 2000), *P. ananatis* and *P. agglomerans* isolated from human hosts (De Beare et al. 2004; Kratz et al., 2003), and *Pantoea* species from the environment (Loiret et al., 2004; Torres et al., 2005). Coutinho et al. (2002) showed that bacteria causing bacterial blight and dieback of *Eucalyptus* are *P. ananatis*, by, among other methods, the analysis of the 16S rRNA gene.

The 16S rDNA sequence is sometimes inadequate for the identification of closely related bacterial species because of: possible lateral transfers within the gene, the highly conserved nature of the gene and multiple copies of the 16S rRNA gene within a single cell (Broughton, 2003; Cillia et al., 1996; Keswani and Whitman, 2001). Alternatively, several other genes have been examined for their potential as phylogenetic markers. These include the *recA* gene involved in recombination and DNA repair (Waleron et al., 2002), the *groE*, which encodes stress proteins (Harada and Ishikawa, 1997), and the *gyrB* gene encoding the ATPase domain of DNA gyrase (Dauga, 2002).

MULTI-LOCUS SEQUENCE ANALYSIS (MLSA)

Analysis of a single gene sequence does not always lead to correct identification of a bacterial isolate. Van Berkum et al. (2003) pointed out that phylogeny of a gene is phylogeny of that gene, not of its host. The authors observed inconsistency in the 16S rRNA, ITS and 23S rRNA-derived phylogenetic relationships among bacterial strains of *Rhizobiaceae*. Fukushima et al. (2002) evaluated the *gyrB* and 16S rRNA sequences of *Salmonella, Shigella* and *Escherichia coli* for identification purposes. The classification of bacterial strains obtained by the 16S rRNA sequences analysis was different from that determined by the analysis of the gyrB gene sequences.

“The ad hoc committee for the re-evaluation of the species definition in bacteriology” proposed that not one, but at least five genes sequences of any given strain should be analysed, to obtain a phylogenetic data for species identification (Stackebrandt et al., 2002). Such genes should be: protein coding (housekeeping genes), located at diverse chromosomal loci, present in a single copy, not prone to recombination and widely distributed among bacterial taxa (Stackebrandt et al., 2002; Zeigler, 2003).

The multi-locus sequence analysis (MLSA), a “method for the genotyping characterisation of diverse groups of prokaryotes” (Gevers et al., 2005), stems from the multi-locus sequence typing (MLST), a “method for the genotyping characterisation of prokaryotes at the infraspecific level” (Gevers et al., 2005). MLST was developed to identify hypervirulent lineages of *Neisseria meningitides* (Maiden et al., 1998). This approach is used in clinical epidemiology for molecular typing of strains belonging to a single, described species (Gevers et al., 2005; Singh et al., 2006). The purpose of MLSA is to select the genes, which combined sequences, when analysed, could clearly divide the strains belonging to a genus into separate, previously described species. The division of strains into species obtained by MLSA should be congruent with other methods, specifically with the DNA-DNA hybridisation (Gevers et al., 2005).

Zeigler (2003) compared 49 sequences of complete bacterial genomes belonging to 23 described species to find the genes valuable for predicting relatedness of whole genomes in bacteria. He selected 32 protein-encoding genes applying the following criteria: genes were

present in all 49 genomes, only one copy of the gene was present in each of the genomes, the genes were "unique" (no close paralogues to confuse analysis), the gene sequence had to be long enough to include informative phylogenetic data and short enough for routine sequencing. The 16S rRNA gene was included as the most popular in phylogenetic studies. From the pool of 32 protein-coding genes, only eight showed an excellent ability for a species prediction. The best two genes for identification of diverse bacteria to a genus and species level were *rec*N, a recombination and repair protein-encoding gene and *dna*X, a gene encoding two subunits of DNA polymerase III. The 16S rRNA gene sequence had the poorest ability in assigning bacterial strains to the correct species.

Naser et al. (2005) examined the usefulness of RNA polymerase α subunit (*rpo*A) and phenylalanyl –tRNA synthase (*phe*S) gene sequences as a tool for identification of enterococci. Ninety-six representative strains comprising all currently described *Enterococcus* species were analysed. All species were clearly differentiated based on their *rpo*A and *phe*S sequences. The author concluded that these two genes could be successfully used for identification of *Enterococcus.* Richter et al. (2006) sequenced seven genes of various strains within *Borrelia burgdorferi sensu lato.* Only the strains for which DNA-DNA hybridisation values were available in the literature were selected. The delineation obtained with MLSA was fully congruent with that established by DNA-DNA reassociations. The results also revealed an existence of a novel species, *Borrelia spielmanii* sp. nov.

INTEGRATION OF VARIOUS METHODS FOR A POLYPHASIC IDENDIFICATION

Identification means assigning an unknown bacterial strain into one of the existing taxonomic groups (Vandamme et al., 1996). The basic unit of bacterial taxonomy is the species.

> The species is defined as "a group of strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m" (Wayne et al., 1987).

This definition is regarded the "golden rule" in delineation of bacterial species (Vandamme et al., 1996). However, the DNA-DNA hybridisation is difficult to perform, slow, reproducibility between laboratories is questionable, the data cannot be stored in databases

and each experiment requires the inclusion of sometimes several reference strains (Stackebrandt, 2002). Rosselló-Mora and Amann (2001) provided more universal and not as rigid definition:

> The species is “a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property” (Rosselló-Mora and Amann, 2001).

The definition calls for using a polyphasic approach, including both phenotypic and genotypic techniques, to identify a bacterial strain to the species level. “The ad hoc committee for the re-evaluation of the species definition in bacteriology” suggested the following components should be considered for species delineation (Stackebrandt et al., 2002): the 16S rRNA gene sequence (>1,300 bp), protein-coding gene sequences, genotyping using fingerprinting techniques targeting whole genomes (PFGE, RAPD, rep-PCR, AFLP), phenotypic characterisation using preferably commercial systems, MLSA and DNA-DNA reassociation. The polyphasic characterisation is compulsory for the description of novel species in the “International Journal of Systematic and Evolutionary Microbiology”(Kämpfer et al., 2003).

Xanthomonas arboricola pv. *fragariae*, a novel pathovar causing a new disease of strawberry, bacterial leaf blight, was described following polyphasic characterisation (Janse et al., 2001). The authors used a variety of phenotypic techniques, pathogenicity tests, AFLP analysis and DNA-DNA hybridisation to compare the leaf blight-causing strains with reference strains of other *Xanthomonas* species.

Coutinho et al. (2002) used a polyphasic approach to identify the causal agent of bacterial blight and dieback of *Eucalyptus.* Bacterial strains were characterised phenotypically by using the nutritional (Biolog) and physiological (API system) tests and fatty acid analysis. The 16S rDNA sequences were compared with those of other *Enterobacteriaceae* from the GenBank and phylogenetic analysis revealed that the isolates belong to the genus *Pantoea.* DNA-DNA hybridisation between the strains from *Eucalyptus* and the type strain of *P. ananatis* identified the bacterium causing blight and dieback of *Eucalyptus* as *P. ananatis*.

Polyphasic analysis has been used to identify previously undescribed pathogens of passion fruit. Goncalves and Rosato (2000) isolated *Xanthomonas campestris*-like bacteria from passion fruit plants and fruits. The genetic diversity of 55 strains was examined using RAPD, rep-PCR, RFLP of the 16S-23S rDNA intergenic spacer region, PFGE, AFLP and SDS-PAGE of whole cell proteins. *Xanthomonas* strains from passion fruit could not be classified by the analysis of these data. Only DNA-DNA hybridisation identified them as *X. axonopodis* pv. *passiflorae*.

CONCLUSIONS

A pure culture of the causal agent of the disease is essential for its identification. Isolation of *Pantoea ananatis* from onion seed on the general growth media such as nutrient agar, although possible, is difficult, due to abundant growth of other bacteria and fungi present in seed (Walcott et al., 2002). The development of an appropriate semi-selective medium would increase the efficiency of detection of *P. ananatis* in onion seed and other plant material.

Pantoea strains should be classified and identified based on genotypic and phenotypic characteristics, as identification based on phenotypic tests only does not always lead to clear results (Gavini et al., 1989, Mergaert et al., 1993).

PFGE is considered as the most-discriminatory DNA-based typing method (Struelens et al., 2001). Although it was used successfully to distinguish *P. stewartii* subsp. *stewartii* from related *Pantoea* species (Coplin et al., 2002), this technique is laborious and time consuming (Olive and Bean, 1999). RAPD fingerprints assays are quicker and easier to perform that PFGE. Unfortunately, the assay is difficult to standardise and the RAPD fingerprints are not always reproducible. Additionally, the RAPD primers that generate the best DNA patterns for identification and differentiation must be determined empirically for each taxon.

Rep-PCR and AFLP genomic fingerprinting are proven as reliable methods, with a high degree of reproducibility, for the identification and diagnosis of plant pathogenic bacteria. The discriminatory power of AFLP is equal to that of PFGE (Lindstedt at al. 2000) and the rep-PCR is only slightly less discriminatory than PFGE (Weigel et al., 2004). The genotypic and phylogenetic relationships among microorganisms obtained by the analysis of the AFLP and rep-PCR fingerprints were highly correlated to that obtained using the DNA-DNA

hybridisation studies (Rademaker et al., 2000). These two techniques are easy to perform and relatively inexpensive. However, the results are difficult to compare between laboratories, as the choice of primers, restriction enzymes and gel systems varies between laboratories.

Phylogenetic relationships between bacteria could be determined by comparing the sequences of the stable part of the genes. The gene most commonly used for taxonomic purposes is the 16S rRNA gene. Although this gene has the poorest ability to predict genome relatedness at the species level, compared to 32 protein-encoding genes studied by Zeigler (2003), the 16S rRNA gene sequence is available for a large number of strains of almost all bacterial species. The sequences are deposited in publicly accessible databases. The sequence of an unknown strain is easily compared with many previously deposited sequences, making identification quick and efficient.

MLSA of housekeeping genes has a superior potential for species discrimination than the 16S rDNA analysis (Zeigler, 2003). The analysis of several genes of diverse chromosomal loci has the potential to replace DNA-DNA hybridisation in the delineation of bacterial species (Gevers et al., 2005; Richter et al., 2006; Zeigler, 2003). MLSA is simple and portable between laboratories, but the cost of sequencing several gene fragments for each isolate makes it rather a distant option for the routine identification of bacteria.

One technique is not sufficient for identification of plant pathogenic bacteria especially those causing previously undescribed diseases of new hosts. The identification of such bacteria requires a polyphasic approach, employing several genotypic and phenotypic methods (Alvarez, 2004; Coutinho et al., 2002; Vandamme et al., 1996).

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CHAPTER 2

PA 20, a Semi-Selective Medium for Isolation and Enumeration of *Pantoea ananatis*

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Abstract

A semi-selective medium, PA 20, was developed for the isolation and enumeration of *Pantoea ananatis* from plant material, specifically from onion seed. The medium has a pH of 8.0 and contains $NH_4H_2PO_4$, K_2HPO_4, magnesium sulphate, NaCl, D (+) arabitol, crystal violet, bromothymol blue and thallium nitrate. All *P. ananatis* strains from a variety of hosts produced characteristic yellow colonies in 6-7 days at 25°C. Plating efficiencies on PA 20 in comparison to nutrient agar ranged from 92 to 112%. Recovery from naturally infested and artificially contaminated onion seed was high, with an almost total reduction of saprophytes.

Keywords: PA 20 medium; *Pantoea ananatis*, Seed; Selective isolation

1. Introduction

Pantoea ananatis is a pathogen causing diseases in a number of economically important plants including onion (Gitaitis and Gay, 1997; Schwartz and Otto, 2000), *Eucalyptus* (Coutinho et al., 2002), corn (Paccola-Meirelles et al., 2001), melons (Bruton et al., 1986; Wells et al., 1987), sudangrass (Azad et al., 2000), rice (Azegami et al., 1983) and pineapple (Serrano, 1928).

P. ananatis is both seed-borne and seed-transmitted in sudangrass (Azad et al., 2000), rice (Tabei et al., 1988) and onions (Walcott et al., 2002). The disease of onion, caused by *P. ananatis*, was named center rot. Onion seed associated with the first outbreak of center rot in Georgia, USA (Gitaitis and Gay, 1997), was produced in South Africa and it was suspected that the bacterium was introduced into that country on infected seed lots (Walcott et al., 2002). In South Africa, efforts to control the quality of commercially produced onion seed mainly focus on the detection of fungal pathogens, and little is known about potential bacterial pathogens associated with these seeds. Nutrient Agar (NA) and yeast extract dextrose calcium carbonate (YDC) (Wilson et al., 1967) are the common growth media used to isolate *P. ananatis* from plant material and seed (Azad et al., 2000; Coutinho et al., 2002; Walcott et al., 2002). These media, however, are non-selective and many other organisms present as saprophytes or endophytes in plant material and in seed may hamper the detection of the target pathogen. In this paper, we describe a semi-selective medium, PA 20, which suppresses growth of many saprophytic microorganisms and serves as a suitable medium for growth and enumeration of *P. ananatis*. The medium was specifically developed to detect this pathogen on onion seed.

2. Materials and Methods

2.1. Bacterial strains

Bacterial strains used in this study are listed in Table 1. Stock cultures of all isolates were maintained in milk-glycerol liquid medium (10% skim milk, 15% glycerol in distilled water) at –70°C. Stock strains were transferred onto NA (Difco) plates and incubated at 25°C to recover growing cultures. Cultures were routinely checked for purity and colony characteristics.

2.2. PA 20 development

Criteria for the semi-selective medium were: differentiation between *P. ananatis* and other bacteria by colony morphology and inhibition of fungal and bacterial contaminants commonly found on onion seed, without hampering the growth of *P. ananatis*.

Substrate utilisation profiles using the BIOLOG (BIOLOG, Inc., Hayward, CA) plates designed for gram negative bacteria revealed that *P. ananatis* strains utilised D (+) arabitol, while the majority of other bacteria listed in Table 1 did not (data not shown).

A variety of media, based on the utilisation of D (+) arabitol and modification of Medium C of Dye (Dye, 1968) were prepared. Different concentrations of NaCl: 0.5; 1.0; 2.0; 3.0 and 4.0%, and a range of pH from 7.0 until 10.0 were evaluated. The pH indicator bromothymol blue was added to monitor pH changes during bacterial growth. Crystal violet and thallium nitrate were added (Ishimaru and Klos, 1984) to suppress other bacteria and fungi (Norris et al., 1976; Srivastava et al., 1976). A loopful of bacterial suspensions of the strains listed in Table 1 was streaked on the media in triplicate and observed daily for bacterial growth.

The medium that satisfied the criteria specified above was named PA 20 and had the following composition per litre: NaCl, 20 g; K_2HPO_4, 1 g; $NH_4H_2PO_4$, 1 g; $MgSO_4 \times 7H_2O$, 0.2 g; bromothymol blue, 1 ml of 1.6% aq. solution; crystal violet, 2 ml of 0.075% aq. solution; agar, 15 g. The pH was adjusted to 8.0 with 1.0 N NaOH. After autoclaving and cooling to 50° C, 3 g of D (+) arabitol dissolved in 5 ml water and 2 ml of 1% aq solution of thallium nitrate were added. Both solutions were filter sterilised. Plates were stored at room temperature for 24 hours before use.

2.3. Colony morphology and plating efficiency on PA 20

Sixteen *P. ananatis* strains were selected to determine colony morphology and plating efficiency on PA 20 (Table 2). The strains were grown on NA for 24-48 hours at 25^oC. Colonies were suspended in 9 ml quarter-strength, sterile Ringer (Oxoid) buffer (RB) to an absorbance density of 0.1 at 640 nm (UV-160A Shimazu spectrophotometer). Ten-fold serial dilutions were prepared in half-strength nutrient broth (Difco), and 0.1 ml of selected dilutions was spread-plated in triplicate on PA 20 and a non-selective NA (contained per litre: beef extract, 3.0 g; peptone, 5.0 g; agar, 15.0 g; pH 6.8 ± 0.2 at 25^oC). Plates were incubated at 25^oC. Colonies were assessed and counted after 7 days. Plating efficiencies

were determined by dividing the number of colonies growing on PA 20 by the number of colonies on NA, and multiplying the quotient by 100. The experiment was replicated twice.

2.4. *PA 20 selectivity assessment*

Selectivity was evaluated by assaying pathogen-free onion seed artificially infested with *P. ananatis* strain Pans 2002-2. One seed lot contained a large number of saprophytic bacteria (5.4 x 10^6 CFU/gram) while the second seed lot had a low population of saprophytes (2.2 x 10^3 CFU/gram). For each seed lot two sub-samples, 5 g each, were placed in separate sterile mortars and crashed with pestles. Crashed seeds were transferred to separate sterile Erlenmeyer's flasks containing 100 ml of RB. One of the sub-samples of each seed-lot was spiked with a suspension of *P. ananatis* to produce an estimated population of 10^3 CFU/ml. Flasks were incubated for 30 min at room temperature on a rotary shaker. Ten-fold serial dilutions were made in half-strength nutrient broth, and 0.1 ml of each dilution (spiked and non-spiked) was spread on the surface of PA 20 and NA. Colonies were counted after 7 days incubation at 25°C. Suspected *P. ananatis* (selection based on colony morphology), were purified and their identities confirmed by: oxidase test, indole production and Hugh-Leifson oxidation/fermentation (Walcott et al., 2002). Tests were replicated three times.

2.6. *Isolations from naturally infested seed*

Five onion seedlots obtained from seed companies and farmers were tested for the presence of *P. ananatis*. Seed extracts were prepared as described above. For each sample, ten-fold dilution series were made in half-strength nutrient broth, and 0.1 ml of each dilution and direct seed extract was plated in triplicate on NA and PA 20. Plates were incubated at 25°C and examined daily for the presence of characteristic *P. ananatis* colonies. Three suspected *P. ananatis* isolates were selected from each PA 20 plate (selection based on colony morphology) for further identification.

2.7. *Identification of* P. ananatis *from naturally infested seed*

Isolates were purified and their identities confirmed by pigment production on NA, motility, cell morphology, Gram stain, oxidase, catalase, indole production, Hugh-Leifson oxidation/fermentation, gas production from glucose and hydrogen sulphide from cysteine

(Mergaert et al., 1993). The tests were done according to methods described by Fahy and Hayward, 1983.

Pathogenicity was determined on onion (*Allium cepa,* cv. Granex 33) in greenhouse assays by using a stub inoculation test adapted from Fenwick and Guthrie, 1969. A sterile needle was dipped into the bacterial colony on NA (24-48 hours growth) and then the needle was inserted under the epidermis of a leaf. At least two leaves were inoculated per isolate. Inoculated plants were incubated in a greenhouse with 27°C/23°C day/night temperatures and observed daily for the development of symptoms.

3. Results and discussion

3.1. PA 20 development

D (+) arabitol as a single carbon source, the pH, NaCl concentration and presence of thallium nitrate all contributed to the PA 20 selectivity. *P. ananatis* grew on PA media at pH 7.0; 8.0 and 9.0, but some saprophytes grew as well, sometimes producing large, slimy colonies. The addition of thallium nitrate inhibited saprophytic bacteria at pH 9.0 but not at pH 7.0. However, the growth of *P. ananatis* on PA medium at pH 9.0 containing thallium nitrate was also hampered. Only at pH 8.0 with thallium nitrate did *P. ananatis* grow well and saprophytes listed in Table 1 were inhibited. The concentration of NaCl influenced the colony size and the time of growth to achieve a colony large enough for recognition and enumeration. When the concentration of NaCl was lower or higher than 2% colonies of *P. ananatis* were either too small for evaluation (<2%) or took 10 or more days to appear on the media (>2%). Thus the medium, named PA 20, containing thallium nitrate, 2% NaCl and a pH 8.0 was selected for further evaluation.

3.2. Colony morphology on PA 20 and plating efficiency

All strains of *P. ananatis* grew on PA 20 medium and colonies were visible after incubation for 6 to 7 days (Table 1). Colonies were yellow, 3-4 mm in diameter; shiny, drop-shaped with small, granular, darker yellow inclusions within. *P. ananatis* decreased the pH of the medium (degradation of D (+) arabitol to acid) and produced a diagnostic yellow zone around individual colonies (Fig. 1).

*Pseudomonas syringae, Pantoea agglomerans, Pectobacterium carotovorum*_subsp. *carotovorum, Xanthomonas campestris* and saprophytes isolated from onion seed did not grow on PA 20 medium. *Pantoea stewartii* subsp. *indologenes* produced small, 1-2 mm in diameter, yellow colonies with a dark green centre (Table 1).

Plating efficiencies of the 16 *P. ananatis* strains ranged from 92% to 112% with a mean of 99.5% on PA 20 medium compared to NA. Results are presented in Table 2.

3.3. PA 20 selectivity assessment

P. ananatis was not recovered from seed samples plated on NA or from non-amended seeds on a semi-selective PA 20. Plates of NA were overgrown by other bacteria and fungi. In contrast, on PA 20, saprophytic growth was reduced by 99-100% (Table 3, Fig. 2A and 2B) and *P. ananatis* was easily identified by characteristic, yellow, drop shaped colonies (Fig. 2B), that were all oxidase negative, indole positive and glucose fermentative. *P. ananatis* was recovered at the expected concentrations on PA 20 from both amended seed samples. The results are presented in Table 3.

3.4. Recovery from naturally infested seed

P. ananatis was isolated on PA 20 medium from two of the five onion seed samples obtained from seed companies and farmers. The selection of suspected *P. ananatis* on NA was difficult, because many saprophytes produced similar yellow colonies. The detection on PA 20 was not hampered, because other microorganisms present in onion seed either did not grow on the medium or had distinctly different colony morphology.

All suspected *P. ananatis* purified from PA 20 produced yellow colonies on NA, were gram-negative rods (1.5-2.0 μm length and 0.5-0.75 μm width), motile, oxidase negative, catalase and indole positive, and fermentatively utilised glucose. They did not produce gas from glucose and hydrogen sulphide from cysteine. The results of tests are characteristic for *P. ananatis* as described by Mergaert et al. (1993).

Pathogenicity of *P. ananatis* to the onion cultivars Granex 33 is shown in Table 1. All pathogenic isolates induced identical symptoms on onion leaves. Two to four days after inoculation water soaked spots appeared on leaves that expanded into longitudinal, bleached-green lesions with chlorotic margins.

In conclusion, PA 20 is a useful semi-selective medium for the isolation and enumeration of *P. ananatis* from onion seed. Experiments demonstrated the efficiency of the medium in recovering *P. ananatis* from seed containing large number of other microorganisms. The medium should not, however, be used as a single diagnostic tool. *P. ananatis* strains non-pathogenic to onion are indistinguishable from pathogens on PA 20 (Table 1, Table 2). Identity of the pathogen must be confirmed using selected phenotypic characteristics and by conducting pathogenicity tests described in this paper. *P. ananatis* from hosts other then onion also grew on PA 20, showing its potential as a general-purpose medium for isolation of these bacteria.

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Table 1. Bacterial strains [a] used in this study, their growth on PA 20 medium and their pathogenicity to onion (*Allium cepa*) cv. Granex 33.

Bacterium and strain number	Host	Growth on PA 20 [b]	Pathogenicity to onion in a stub-inoculation test [c]
P. ananatis			
LMG 2665[T]	*Ananas cosmosus*	+	-
LMG 2676	*Puccinia graminis*	+	-
LMG 20103, 20104	*Eucalyptus*	+	-, -
ATCC 35400	*Cucumis melo*	+	-
ATCC BAA 515	*Allium cepa*	+	-
Blackshank 15, 24, 30	*Allium cepa*	+	+, -, -
Hort. Hill 7, 24, 31, 32			-, +, +, +
Pans 99-8, 99-23, 2002-2			-, -, +
BD 321, 322, 323,234,325, 326, 327, 328, 329	*Allium cepa*	+	all -
BD 377, 381, 383, 386, 387, 388, 390, 392, 393	*Allium cepa*	+	all +
P. agglomerans			
LMG 1286[T]	knee laceration	-	-
SUH 2	*Allium cepa*	-	+
DAR 49828	*Allium cepa*	-	-
P. stewartii subsp. *indologenes*			
LMG 2632[T]	*Setaria italica*	+ [d]	-
LMG 2671	*Ananas cosmosus*	+ [d]	-
Pseudomonas syringae	*Allium cepa*	-	not tested
Pectobacterium carotovorum subsp. *carotovorum*	*Solanum tuberosum*	-	not tested
Xanthomonas campestris pv. *allii*			
BD 142, 143, 211	*Allium cepa*	-	not tested
Saprophytes, 22 isolates	*Allium cepa* seed	-	all -

[a] LMG: BCCM/LMG Culture Collection, Universiteit Gent, Belgium; ATCC: American Type Culture Collection, Manassas, VA; DAR: Australian Collection of Plant Pathogenic Bacteria, Orange; BD: Plant Pathogenic and Plant Protecting Bacteria (PPPPB), ARC-PPRI, South Africa; Blackshank, Hort. Hill and Pans: R. Walcott, Department of Plant Pathology, University of Georgia, Athens; Saprophytes from onion seed: this study.

[b] Growth on PA 20 medium from suspension of cells plated for isolated colonies.

[c] A sterile needle was dipped into the bacterial colony on NA (24-48 hours growth) and then the needle was inserted under the epidermis of an onion leaf; results recorded after one week; pathogenic (+), non-pathogenic (-), not tested.

[d] Grew on the medium but colony morphology was different from *P. ananatis*.

Table 2. Plating efficiency of *P. ananatis* strains on PA 20 medium relative to NA medium.

Strain no	Number of CFU [a] on:		Plating efficiency
	NA	PA 20	(%) on PA 20[b]
Strains from onion			
ATCC BAA 515	24.6	22.8	92.6
BD 325	40.6	40.8	100.4
BD 327	13.3	13.2	100
BD 377	42	43	102
BD 387	29	28	96.6
BD 390	29	30	103.4
Blackshank 15	29.7	31	104.5
Hort. Hill 24	54.4	51.6	95
Hort. Hill 31	42.6	43.9	103
Pans 99-8	28	25.8	92
Pans 2002-2	44.6	50	112.1
		Average	100.1
Strains from other hosts			
ATCC 35400	27	24	92
LMG 2665^T	26.2	24.2	92.4
LMG 2676	23.2	25.4	109.5
LMG 20103	25.8	26	100
LMG 20104	33.8	33.6	100
		Average	98.8
TOTAL AVERAGE			99.5

[a] Strains were pre-cultured on NA medium 24-48 hours at 25°C, colonies removed with sterile cotton swab and suspended in SR to an absorbance density of 0.1 at 640 nm; tenfold dilution series were prepared in half-strength nutrient broth and 0.1 ml volumes were plated on test media and incubated at 25°C for 7 days. Figures in each row are from the same dilution.

[b] Plating efficiency = colony forming units on PA 20 medium/colony forming units on NA x 100. Figures were calculated from the mean colony numbers of minimum five plate counts per strain.

Table 3. Recovery of *P. ananatis* on a semi-selective medium PA 20 from two pathogen-free onion seed lots spiked with Pans 2002-2 to a final concentration ~103 CFU/ml. NA was used as a control, general growth medium. Characteristic yellow, drop shaped colonies on PA 20 (Fig. 1) were considered as *P. ananatis*.

Seed sample [a]	CFU/ml [b] recovered on			PA 20 evaluation
	NA medium	PA 20 medium		
	total CFU/ml	total CFU/ml	*P. ananatis* CFU/ml [c]	
not amended				% reduction saprophytes
A	105	0	0	100
B	270000	41	0	99
amended				% *P. ananatis* recovery
A	4220	4198	4197	100
B	275000	3130	3080	98

[a] A - seed with low (2.2 x 10^3 cfu/gram) population of saprophytes and B - seed with high (5.4 x 10^6 cfu/gram) population of saprophytes. Not amended seed - 5 gram seed crashed with mortar and pestle, 100 ml of quarter-strength Ringer (Oxoid) buffer; amended seed - as above plus a bacterial suspension of strain Pans 2002-2 to a final concentration ~10^3 CFU/ml were mixed and incubated for 30 min at room temperature on a rotary shaker.

[b] Values are the mean of three replications. Five ten-fold serial dilutions of each seed extracts were made in half-strength nutrient broth, 0.1 ml of each dilution and direct seed extract was plated on three plates of each of PA 20 and NA. Colonies were counted after 7 days incubation at 25°C.

[c] Identity of two suspected *P. ananatis* colonies from each PA 20 plate confirmed by physiological tests (oxidase negative, indole positive, glucose fermentative.

Fig. 1. Colony morphology of *P. ananatis* on PA 20 medium after 7 days of incubation at 25°C. Colonies are yellow, 3-4 mm in diameter, shiny, drop shaped with small, granular, darker inclusions inside. Lighter zones around colonies on PA 20 are yellow and contrast with the dark blue of the medium.

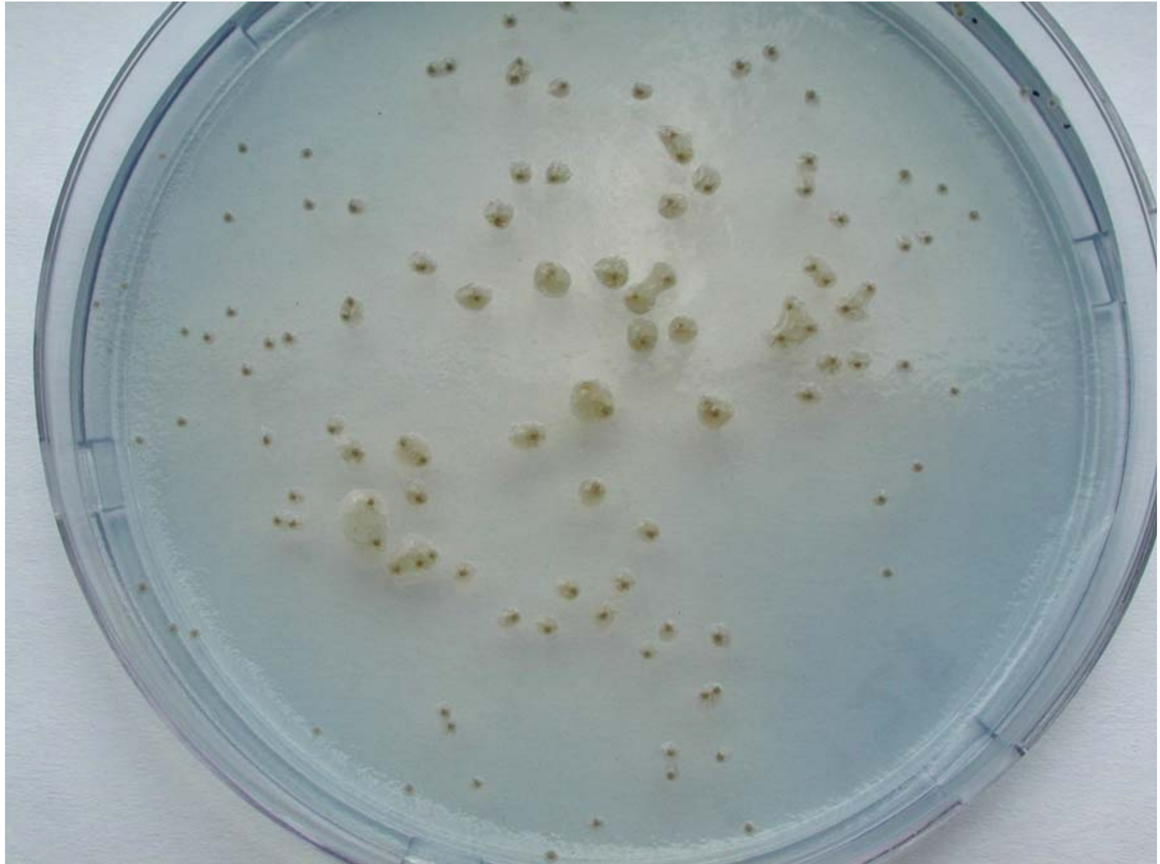

Fig. 1

Fig. 2. Comparison in selectivity between non-selective NA (left) and semi-selective medium PA 20 (right). Isolations were done from: A – *P. ananatis*-free onion seed; B – the same seed as in A spiked with *P. ananatis*. Note total suppression of non-target microflora on PA 20.

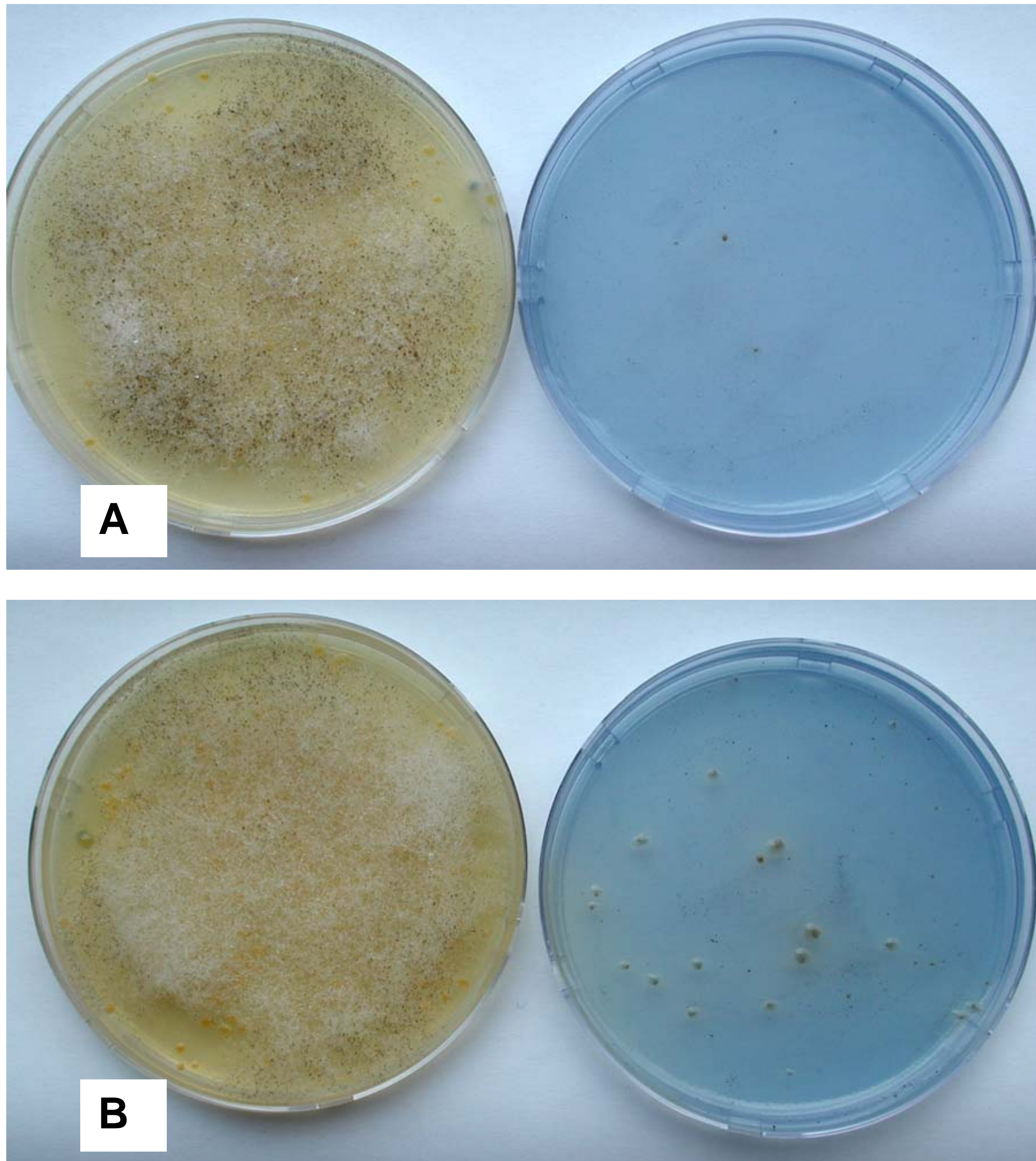

Fig. 2