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Short communication

An efficient microtiter system to determine *Agrobacterium* biovar

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Abstract

A microtiter system for biovar characterization of *Agrobacterium* strains which simplifies the analysis of a large number of isolates is described. This method is based on incubation of bacterial strains in microplate wells previously amended with media specifically used by the different *Agrobacterium* biovars. More than 150 purified *Agrobacterium* strains isolated from the most common host plants were analysed by the microtiter system. It proved to be an excellent tool using less reagents, time and space for incubation in comparison with the traditional method.

The *Agrobacterium* genus is composed of ubiquitous bacteria that can be found in a variety of environments like soil, plants, water (Lippincot, 1981; Moore and Canfield, 1996) and occasionally even in humans (Southern, 1996; Yu et al., 1997). Some agrobacteria are important phytopathogenic agents capable of infecting a large spectrum of host plants (De Cleene and De Ley, 1976) and introduce a part of their Ti or Ri plasmids into the plant chromosome (Agrios, 1988). The presence of tumours in plants after this transformation reduces their economic value (Kennedy, 1980). The ability of *Agrobacterium* to transfer DNA into crop plants is now being used for plant transformation and some strains of this bacterium have become important biotechnological tools (Hooykaas and Schilperoort, 1992). Nevertheless, the presence of living *Agrobacterium* cells in transformed plants before their release, and the need to avoid dissemination of genetically manipulated organisms by means of transgenic plants, is a risky affair (Matz et al., 1996). Consequently, the economic and ecological threat that *Agrobacterium* poses has prompted a search for information regarding its diagnosis, identification and characterization. The genus *Agrobacterium* has been classified into biovars based on phenotypic characteristics including the ability to metabolize specific substrates to the bacteria (Kerr, 1992). Biovar classification coincides with the latest proposal for the division of genus into species, demonstrating the importance of these biochemical criteria (Sawada et al., 1993). As neither the serological nor molecular data available correlate exactly with biovar classification, biochemical testing is still necessary. Although sensitive detection methods for some *Agrobacterium* species have been established (Cubero et al., 1999), classification into biovars has not been improved since early reports (Moore et al., 1988).

The most common diagnostic tests to separate strains of *Agrobacterium* into three biovars include the production of 3-ketolactose, acid production from sucrose, erythritol or melezitose, and alkali production from malonic, L-tartaric, propionic or mucic acids, the growth and pigmentation in ferric ammonium citrate, and citrate utilization (Moore et al., 1988). Other tests proposed are oxidase reaction, L-tyrosine utilization and bacterial growth at 35 °C and in 2% NaCl (Moore et al., 1988). The major drawbacks to traditional biovar

Table 1. Test selected to determine *Agrobacterium* biovars and expected results[1]

	Biovar 1	Biovar 2	Biovar 3
3-ketolactose production	+	−	V[2]
Citrate utilization	V−	+	+
Ferric ammonium citrate	+	−	−
Alkali from malonate	−	+	+
Acid from			
Sucrose	+	−	V
Melezitose	+	−	−
Alkali from			
L-tartaric	−	+	+
Mucic acid	−	+	−

[1]Most of the results can be obtained after 48 h. For some slow growing strains additional time may be required to obtain a reliable result.
[2]V: variable; V−: most of the strains are negative for this test.

classification are the lengthy preparation time for the test media, the incubator space required and lastly a considerable amount of reagents. This, plus the time needed to obtain results and the frequent risk of contamination, makes it difficult to study a large number of strains simultaneously, as is the case with epidemiological, certification or sanitation studies.

In this work we suggest that the use of microplates to determine *Agrobacterium* biovars reduces some of the disadvantages of traditional methods. The proposed system uses less reagents, incubator space, and decreases incubation time from 10–15 days to 48–72 h. We selected eight of the proposed tests by Moore et al. (1988), namely citrate utilization, growth and pigmentation in ferric ammonium citrate, alkali production from malonic, L-tartaric, and mucic acids, acid production from sucrose and melezitose, and 3-ketolactose production (Table 1). The first two tests and alkali production from malonic acid were performed in the media previously described (Moore et al., 1988). For the rest of acid and alkali production tests, a basal medium (Ayers, 1919) was adjusted to 0.1% L-tartaric and mucic acids or 1% sucrose and melezitose respectively. Then 150 µl of the specific substance with its basal medium was dispensed in horizontal rows (A–H) on a sterile culture microplate (Nunc™ Brand Products, Denmark) and 15 µl of a bacterial supension of 10^8 cfu ml^{-1} of each strain to be analysed was added to each vertical row (1–12) using a multichannel pipette. In the case of citrate utilization, a solid medium was used and the strain was added by puncture from a 48 h plate culture. After 48 and 72 h the majority of results could be read (Figure 1). Some additional time was required for slow growing strains, such as some of biovar 2, as in row 3 of Figure 1. The capacity to produce 3-ketolactose (Bernaerts and De Ley, 1963) in microplates was also analysed, by filling the wells with 150 µl of melted or liquid medium and adding the strains by puncture or by a suspension. In both cases the Benedict reagent was added after 48 h and the results were detectable in less than 20 min. This test was performed in separated plates to avoid contamination when Benedict reagent was added. Acid production from erythritol was also analysed but this test was discarded because some false positives, corroborated performing the test in tubes, appeared with several biovar 1 strains.

Using this method, 164 pure cultures of *Agrobacterium* from different hosts like walnut, grapevine, cherry, chrysanthemum, rose and peach or from transgenic citrus plants were analysed in a very short time. Correlation was always observed between the expected biovar results and those obtained in microplates (Table 2). We classified all the strains into one of the three biovars described (Figure 1). Only those isolated from walnut trees behaved differently in one test to any of the three biovars described, either because they were atypical biovar 1 strains, or L-tartaric consumers. Utilization of L-tartaric by biovar 1 strains has already been described by other authors who suggested that this could be due to the adaptation of *Agrobacterium* to the host plant (Ridé et al., 2000). The existence of strains showing results different to those described for the three biovars in more than two or three tests or belonging to intermediate biovar was also previously described in *Agrobacterium* (Bouzar and Moore, 1987; Bell and Ramey, 1990) but such strains were not encountered in this study.

The microplate system described for *Agrobacterium* biovar characterization proved to be more useful and easy to handle than the traditional method in test tubes and could be adapted to the recently described classification in phena (Ridé et al., 2000). Similar methodology was used for biovar determination in *Ralstonia solanacearum* (Hayward et al., 1989). In our laboratory a microtiter system is also currently being used for the identification of other bacteria such as *Pseudomonas savastanoi* pv. *savastanoi* and *Erwinia amylovora* using appropriate culture media. This system might well be an important tool in strain characterization and in epidemiology or studies where a large number of bacteria are involved and no commercial characterization kits are available

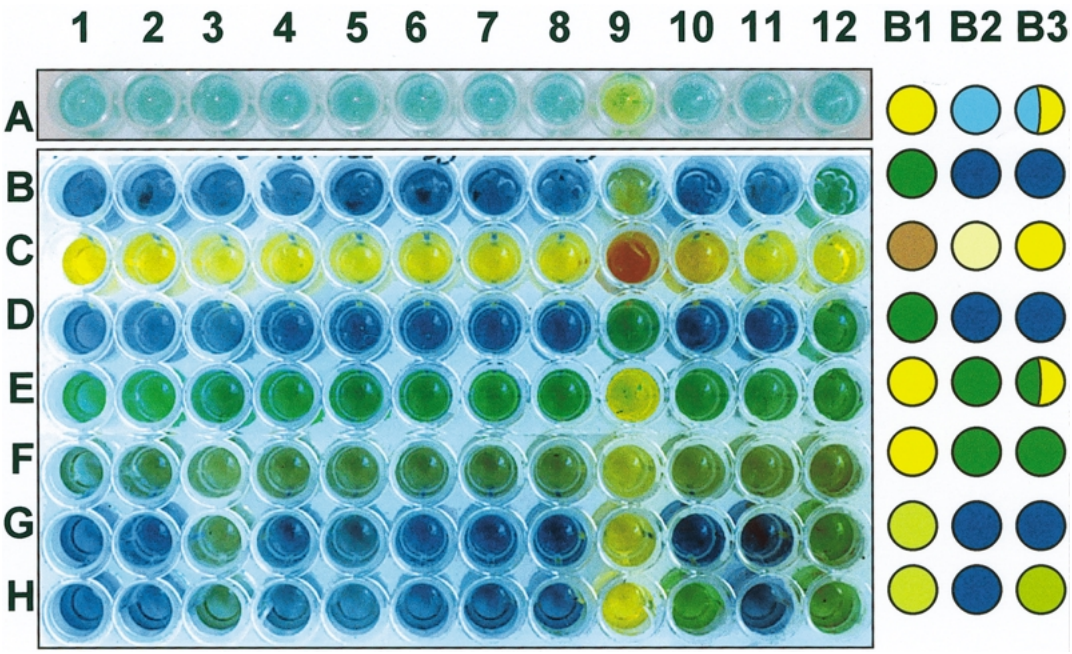

Figure 1. Biovar determination of *Agrobacterium* by microtiter system after 48 h incubation (additional time to obtain reliable results may be required in some test for some slow growing strains like some biovar 2 strains and as observed in row 3). 1–8: pathogenic *Agrobacterium* strains of biovar 2 isolated from a peach tumour; 9: strain C58 of *Agrobacterium* (biovar 1); 10: strain 339-26 of *A. vitis* (biovar 3) isolated from a grapevine tumour; 11: strain K84 of *Agrobacterium* (biovar 2) used in biological control; 12: negative control; A: 3-ketolactose production; B: citrate utilization; C: pigmentation and growth in ferric ammonium citrate; D: alkali production from malonate; E,F: acid production from sucrose and melezitose respectively; G,H: alkali production from L-tartaric and mucic acid respectively. B1, B2 and B3 are expected results for biovar 1, 2 and 3 respectively according to Table 1.

Table 2. *Agrobacterium* strains used and biovar determination

Host	Number of strains	Biovar
Chrysanthemum	11	1
Cherry	40	1
Cherry	27	2
Peach	5	1
Peach	27	2
Raspberry	9	1
Raspberry	15	2
Rose	10	2
Walnut	15[1]	1
Reference strains	*Host*	
C58	Cherry	1
B6	Tomato	1
339-26	Grapevine	3
K84[2]	—	2
Ach5[3]	—	1

[1]Although 15 strains isolated from walnut were L-tartaric consumers, they were considered as biovar 1 strains according to the results of the other tests performed.
[2]*Agrobacterium* strain used in biological control.
[3]*Agrobacterium* strain used for plant transformation and reisolated from transgenic citrus.

as is the case for many species or pathovars of plant pathogenic bacteria. It is a cheap and simple alternative without any equipment requirement and can be adapted for identification or characterization of other bacteria, not only in the field of plant pathogenic bacteria but also for environmental and medical applications.

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