SHORT COMMUNICATION

PHENOTYPIC CHARACTERIZATION OF *PSEUDOMONAS AVELLANAE* (PSALLIDAS) JANSE *ET AL.* AND OCCURRENCE OF COLONY VARIANTS

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SUMMARY

Thirty-seven strains of *Pseudomonas avellanae* from northern Greece, northern Italy and two different areas of central Italy were assessed for some morphological and physiological properties. Whole-cell protein profile analysis was performed by means of UPGMA. The occurrence and induction of transparent colonies of *P. avellanae* sometimes observed on nutrient sucrose agar (NSA) isolation plates, is described. *P. avellanae* strains showed variability in morphological characteristics, gelatin liquefaction and maximum temperature of growth. Cluster analysis of whole-cell protein profiles indicated that strains from northern Italy differ from those from northern Greece and central Italy and that strains from the provinces of Viterbo and Rome clustered in the same group. All strains were negative in ice-nucleation activity and accumulation of poly-β-hydroxybutyrate, and positive in production acid from sucrose. All strains were copper-sensitive. The production of fluorescent pigments on media was influenced by subculture on NSA, and strains from Italy lost this feature after a number of transfers. The existence of populations of the pathogen seems possible. Transparent colonies of *P. avellanae* were also frequently obtained by incubation for three days at supraoptimal growth temperature, by adding 7% sucrose to the nutrient agar and by subculturing 30 day-old colonies on NSA. Such *P. avellanae* colony variants were encapsulated and did not induce hypersensitivity in tobacco leaves.

RIASSUNTO

CARATTERIZZAZIONE FENOTIPICA DI *PSEUDOMONAS AVELLANAE* (PSALLIDAS) JANSE *ET AL.* E COMPARSA DI COLONIE VARIANTI. Trentasette ceppi di *Pseudomonas avellanae* ottenuti da quattro differenti aree geografiche (Grecia settentrionale, Italia settentrionale e due differenti aree dell'Italia centrale) sono stati saggiati per alcune caratteristiche morfologiche, biochimiche e fisiologiche. L'analisi dei profili delle proteine cellulari totali è stata effettuata mediante UPGMA. Inoltre, vengono descritte la comparsa, l'ottenimento e le principali caratteristiche di colonie trasparenti di *P. avellanae*, talvolta osservate sulle piastre d'isolamento (NSA). I ceppi di *P. avellanae* hanno mostrato variabilità nelle caratteristiche morfologiche, nella capacità di liquefare la gelatina e nella temperatura massima di crescita. La comparazione degli elettroforetogrammi ha evidenziato che i ceppi dell'Italia settentrionale formano un gruppo separato dagli altri e che i ceppi della provincia di Viterbo e Roma erano compresi in un altro. Tutti i ceppi risultavano negativi nell'attività di nucleazione del ghiaccio, nell'accumulazione di poli-β-idrossibutirrato e nella produzione di acidità da saccarosio. I ceppi erano sensibili al rame. La produzione di pigmento fluorescente risultava influenzata dal numero di trapianti su NSA e i ceppi isolati in Italia divenivano non fluorescenti dopo alcuni trapianti. L'esistenza di popolazioni del patogeno sembra possibile. Colonie trasparenti sono state ottenute frequentemente mediante incubazione delle colture per tre giorni ad una temperatura di crescita sopraottimale, mediante aggiunta del 7% di saccarosio all'agar nutritivo e dopo trasferimento su NSA di colonie di oltre 30 giorni. Tali varianti di *P. avellanae* risultavano capsulati e non inducevano ipersensibilità in foglie di tabacco.

Key words: Pseudomonas avellanae, hazelnut, protein analysis, colony variants.

On the basis of the 16S rRNA sequence, percentage of DNA similarity, whole-cell protein and whole-cell fatty acid analysis, the causal agent of bacterial canker of hazelnut (*Corylus avellana* L.), formerly known as *Pseudomonas syringae* pv. *avellanae* Psallidas, has been reclassified as *P. avellanae* (Psallidas) Janse *et al.* (Janse *et al.*, 1996). This bacterium causes severe losses to hazelnut orchards in northern Greece (Psallidas, 1987) and central Italy (Scortichini and Tropiano, 1994; Scortichini *et al.*, 1994) and, apparently, in Croatia (Cvi-

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jetkovic-Monti, personal communication). Moreover, it has recently been found in northern Italy (Scortichini and Morone, 1997). Preliminary observations of some phenotypic characteristics of isolates obtained from such different geographic areas revealed variability and, sometimes, the presence of transparent colonies on the isolation plates (Fig. 1). We here report the results of further study of such aspects.

Thirty-seven strains of *P. avellanae* obtained in different years from four geographic areas were used to study some morphological, biochemical and physiological properties as well as to assess their whole-cell protein profiles (Table 1). Cultures were obtained from hazelnut orchards in central and northern Italy (cultivars 'Tonda Gentile Romana' and 'Tonda Gentile delle Langhe', respectively) (Scortichini and Tropiano, 1994; Scortichini *et al*., 1994; Scortichini and Morone, 1997). We also compared strains from northern Greece obtained from cultivar 'Palaz', kindly supplied by Dr. P.G. Psallidas. After isolation and identification, all cultures were lyophilized.

Colony morphology was observed on nutrient agar (NA) (Oxoid) and NA with 5% sucrose (w/v) added (NSA) after 3 and 7 days of incubation at 25 ± 1°C. Preliminary observations on cultures isolated in Italy indicated that, after several subcultures on NSA, the production of fluorescent pigments on King's medium B (KB) (King *et al*., 1954) declined. Since this feature was not observed with the isolates from Greece (Psallidas, personal communication), freshly isolated cultures as well as strains already adapted to the laboratory were streaked on KB and CSGA medium (Luisetti *et al*., 1972) to record the production of fluorescent pigments. The maximum growth temperature was assessed after 24 h of incubation at different temperatures by checking the turbidity in tubes containing 5 ml of 523 medium (Schaad, 1988). To test the ability of the strains to liquefy gelatin, the technique described by Misaghi and Grogan (1969) was followed. Acid production from sucrose was assessed on the medium of Ayers *et al.* (1919) following the technique described by Lelliott and Stead (1987). Ice-nucleation activity of the four *P. avellanae* groups of strains was checked at –5.5 and at –10°C according to the technique described by Lindow (1990). To detect the accumulation of poly-β-hydroxybutyrate, the technique of Pierce and Schroth (1994) was followed. The cultures were streaked on Nile Blue medium (NB). After incubation on NB and observation under UV light at 365 nm, the cultures were also examined by fluorescent microscopy, at a magnification of 1200x. To assess copper-resistance the technique of Zevenhuitnen *et al.* (1979) was followed. Absolute growth inhibition on the agar surface was recorded as sensitivity to copper.

The soluble whole-cell proteins of *P. avellanae* strains were extracted according to Janse *et al.* (1996). Electrophoretic runs were performed in duplicate to confirm reproducibility. After monodimensional SDS-PAGE, the profiles were read with the naked eye and the presence or absence of a particular protein band was converted into binary data. Ten bands useful to discriminate among the strains were chosen for cluster analysis. Similarity coefficients for all pair combinations were determined by Dice's coefficient (Dice, 1945). Protein patterns were clustered by the unweighted average pair group method (UPGMA) using NTSYS version 1.80 software.

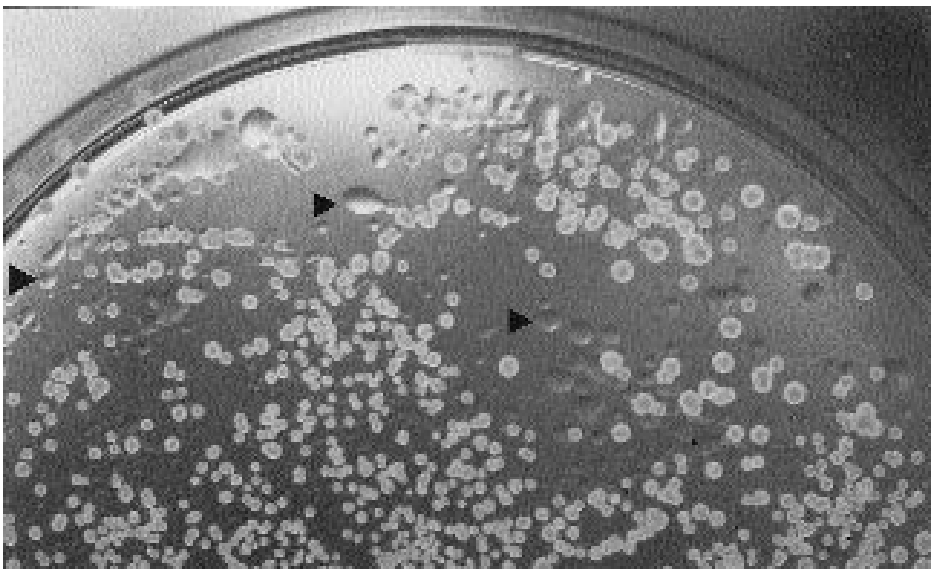


Fig. 1. Occurrence of *P. avellanae* colony variants (arrows) on an NSA plate during the isolation from diseased hazelnut in central Italy (x 1.5).

Table 1. *P. avellanae* strains tested for morphological, biochemical and physiological characteristics and whole-cell protein analysis.

Strain	Country	Province	Year of isolation
BPIC 631	Greece	Drama	1976
BPIC 632	Greece	Drama	1976
BPIC 640	Greece	Kilkis	1976
BPIC 647	Greece	Kilkis	1976
BPIC 665	Greece	Kilkis	1976
BPIC Fl 3	Greece	Kilkis	1976
BPIC 703	Greece	Katerini	1977
BPIC 714	Greece	Kavala	1987
BPIC 1078	Greece	Kilkis	1986
ISPaVe-B-011	Italy	Rome	1991
ISPaVe-B-012	Italy	Rome	1992
ISPaVe-B-013	Italy	Rome	1992
ISPaVe-B-036	Italy	Rome	1993
ISPaVe-B-037	Italy	Rome	1993
ISPaVe-B-056	Italy	Rome	1994
ISPaVe-B-369	Italy	Rome	1995
ISPaVe-B-436	Italy	Rome	1995
ISPaVe-B-439	Italy	Rome	1995
ISPaVe-B-041	Italy	Viterbo	1992
ISPaVe-B-042	Italy	Viterbo	1992
ISPaVe-B-038	Italy	Viterbo	1993
ISPaVe-B-039	Italy	Viterbo	1993
ISPaVe-B-040	Italy	Viterbo	1993
ISPaVe-B-2056	Italy	Viterbo	1994
ISPaVe-B-2057	Italy	Viterbo	1994
ISPaVe-B-2058	Italy	Viterbo	1994
ISPaVe-B-2059	Italy	Viterbo	1994
ISPaVe-B-683	Italy	Viterbo	1996
ISPaVe-B-689	Italy	Viterbo	1996
ISPaVe-B-690	Italy	Viterbo	1996
ISPaVe-B-691	Italy	Viterbo	1996
ISPaVe-B-592	Italy	Cuneo	1995
ISPaVe-B-593	Italy	Cuneo	1995
ISPaVe-B-595	Italy	Cuneo	1995
ISPaVe-B-596	Italy	Cuneo	1995
ISPaVe-B-598	Italy	Cuneo	1995
ISPaVe-B-599	Italy	Cuneo	1995

BPIC: Culture Collection of Benaki Phytopathological Institute, Kiphissia-Athens, Greece.
ISPaVe-B-: Culture Collection of Istituto Sperimentale per la Patologia Vegetale, Roma, Italy.

Colony variants were identified by means of SDS-PAGE of their protein extract and comparison with profiles of *P. avellanae* reference-strains. To obtain the variants, three methods were followed: (*i*) subculturing of the isolates (from Greece and central Italy) grown for 30 days on NSA at 25±1°C (*i.e.* longevity of *P. avellanae* on NSA according to Psallidas, 1993) again on NSA; (*ii*) incubating for 3 days at 30°C (supraoptimal growth temperature) in tubes containing broth medium 523, followed by a streaking on NSA and incubation at 25±1°C; (*iii*) subculturing at weekly intervals for three months, by streaking always from the same NSA plate, a portion of colony on NA containing different percentages of sucrose (1%, 3%, 5%, 7%). To test the viability of the variants on NSA, at intervals of 2, 4 and 6 months the dry NSA was rehydrated by adding some millilitres of sterile saline (0.85% NaCl in distilled water) (SS). Part of the medium was then streaked on freshly prepared NSA and incubated at 25±1°C. Growth of the variants was also checked on the following media: NA, KB, CSGA, GYCA (Van den Mooter *et al.*, 1987), NGA, NDA (Schaad, 1988) and CVP (Cuppels and Kelman, 1974). In addition, ability to induce hypersensitivity was tested by infiltrating a suspension of the variants in SS into tobacco leaves. To verify the presence of the capsule, the cells were first fixed by flame onto a slide. The slide was then placed for 10 min in Indian ink at 55°C, rinsed with tap water, gently dried and observed by phase contrast. Finally, the variants were lyophilized and their viability was tested on NSA after two years.

On NSA, the four groups of *P. avellanae* strains showed some differences in their growth characteristics after 3 and 7 days of incubation at 25±1°C. The strains from Greece were mucoid, glistening with a typical pearl-white colour and with single colonies in the most diluted quadrant of the plate. Strains from central Italy looked more buttery with a creamy-white colour, and also formed single colonies in the most diluted quadrant. On NA, strains from Greece and Italy appeared identical: slow growing, producing very small colonies 0.5 mm in diameter with a creamy-white colour. On this medium, strains from northern Italy tended to show confluent growth in the most diluted quadrant. In addition, radially-striated colonies firstly appeared in strains from Greece and central Italy.

The production of fluorescent pigments varied according to the number of subcultures carried out and to the origin of the strains. On KB, freshly isolated *P. avellanae* strains obtained in Italy showed weaker fluorescence than the isolates from Greece. After several subcultures on NSA, *P. avellanae* strains from Italy no longer fluoresced on KB, whereas strains from Greece,

although less than previously, continued to fluoresce. In all strains the intensity of fluorescence was higher on CSGA than on KB. Again on CSGA the strains from Italy ceased to fluoresce after several subcultures, whereas strains from Greece did not lose this feature.

The maximum growth temperature of strains from Greece and central Italy was 30°C, as opposed to 35°C for the strains obtained from northern Italy. With regard to gelatin liquefaction, *P. avellanae* strains gave a variable response. Strains from Greece and from central Italy did not liquefy gelatin, whereas those from northern Italy gave a variable result, 4 out of 6 being positive. Acid production from sucrose was positive for all strains.

None of the strains from Greece and Italy showed ice-nucleation activity at –5.5 or –10°C or accumulate poly-β-hydroxybutyrate. However, on NB, the strains from Greece started to produce a blue pigment 6 days after incubation, whereas strains from Italy only after 12 days. This characteristic seems to be related to acidification of the substrate inducing change in colour (Pierce and Schroth, 1994). *P. avellanae* strains were sensitive to copper sulphate: they only grew at a concentration of 40 μg ml^{-1}. At 80 μg ml^{-1} growth was quite poor and at 160 μg ml^{-1} no colonies were observed on CYEG.

SDS-PAGE analysis of whole-cell proteins revealed three major groups of *P. avellanae* strains. The strains from northern Italy showed 70% similarity with those from Greece and central Italy. Strains from central Italy and Greece were more homogeneous, forming two clusters defined at 90% similarity level. Moreover, strains from the provinces of Viterbo and Rome (central Italy) clustered in the same group. (Fig. 2).

SDS-PAGE gel electrophoresis of whole-cell proteins, showed six colony variants with a similar profile to a *P. avellanae* reference strain (ISPaVe-B-013 = BPIC 860 = PD 2390) even though the intensity of the bands was very weak. The transparent colony variants (drops of water like colonies) were obtained directly from diseased specimens of hazelnut in central Italy. The appearance of such colonies was not very frequent but when it occurred, a considerable number of variants was observed on NSA plates (Fig. 1). *P. avellanae* colony variants were frequently obtained after restreaking 30 day-old colonies on NSA (Fig. 3a). Finally, colony variants were also induced by incubation in 523 broth medium for 3 days at 30°C (Fig. 3b); after incubation and plating on NSA the percentage of variants obtained varied from 2 to 4%. The streaking on NA containing different percentage of sucrose of three strains (BPIC 631 from Greece, ISPaVe-B-011 from the province of Rome, ISPaVe-B-369 from the province of

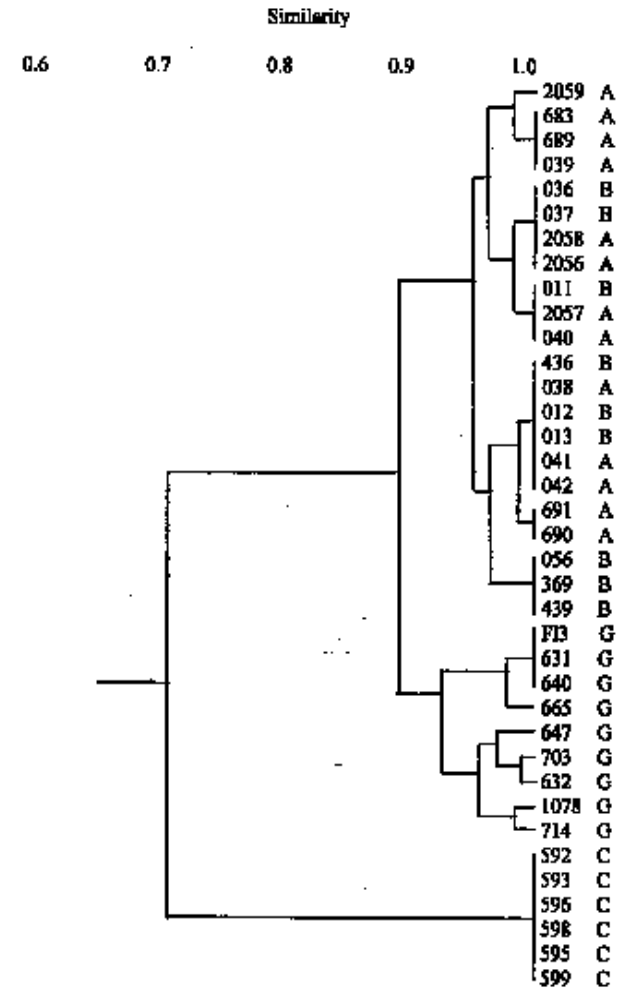
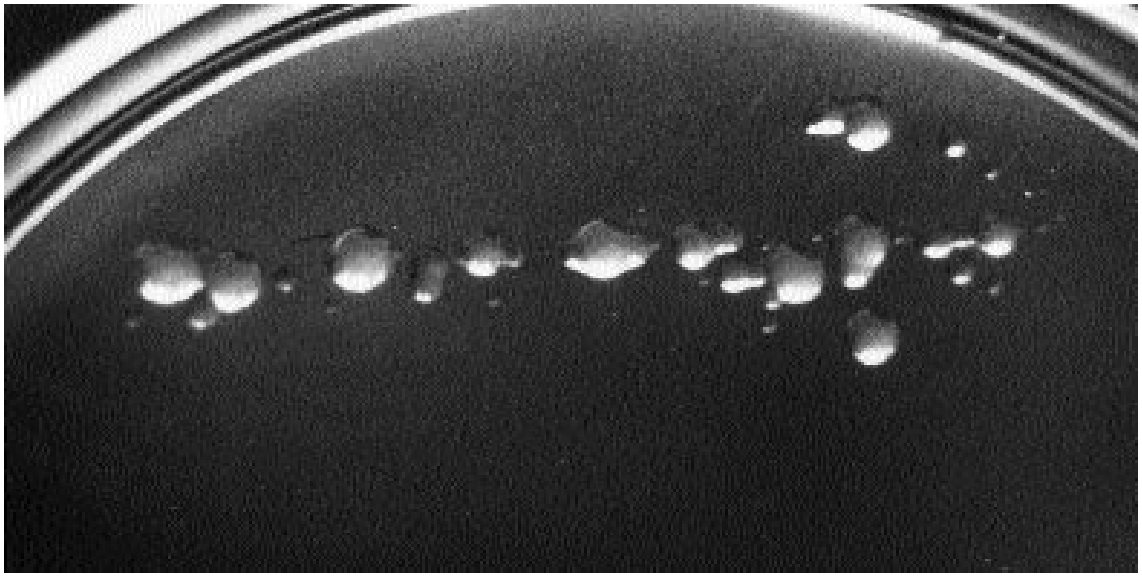
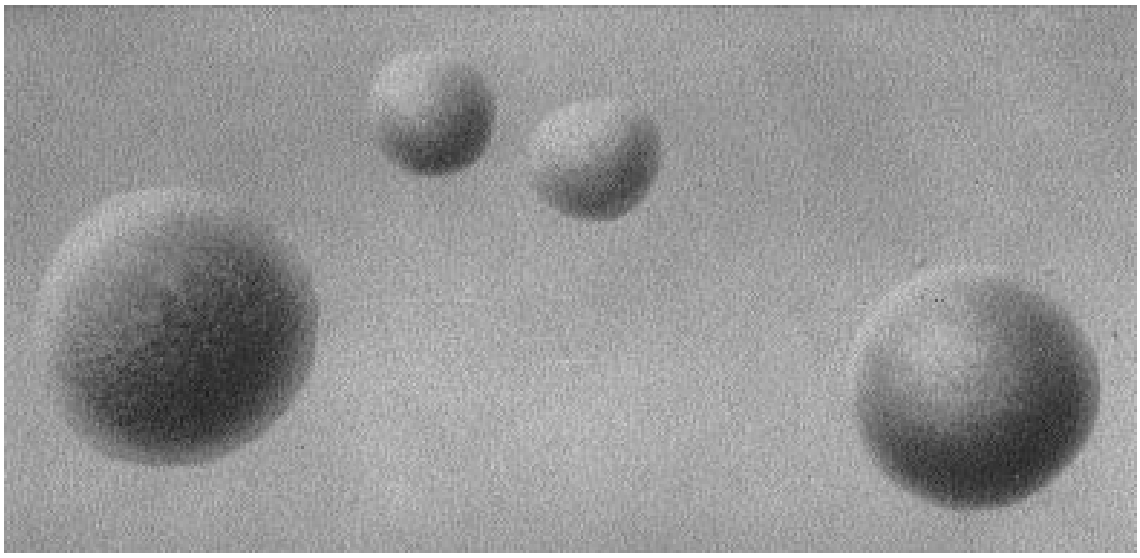


Fig. 2. Dendrogram obtained by UPGMA cluster analysis showing relationships based on whole-cell protein electrophoresis of 37 strains of *P. avellanae* from northern Greece, northern Italy and two different areas of central Italy (see also Table 1). A relationship between the strains and their geographic origin is observed. A) *P. avellanae* strains from Viterbo; B) *P. avellanae* strains from Rome; C) *P. avellanae* strains from Cuneo; G) *P. avellanae* strains from Greece.

Viterbo), always taken from the same NSA plate, gave additional information on the origin of variants. With a sucrose content of 1% *P. avellanae* 011 yielded variants only after 78 days of incubation. With this level of sucrose, during a period of 3 months, other strains did not yield colony variants. With sucrose content at 3%, only the strains from central Italy yielded variants. With 5% sucrose all strains yielded variants after 29 days. At 7% sucrose, the strains from Greece yielded transparent colonies after 8 days, the strains from Italy after 29 days. When subcultured regularly every 7-15 days,



A



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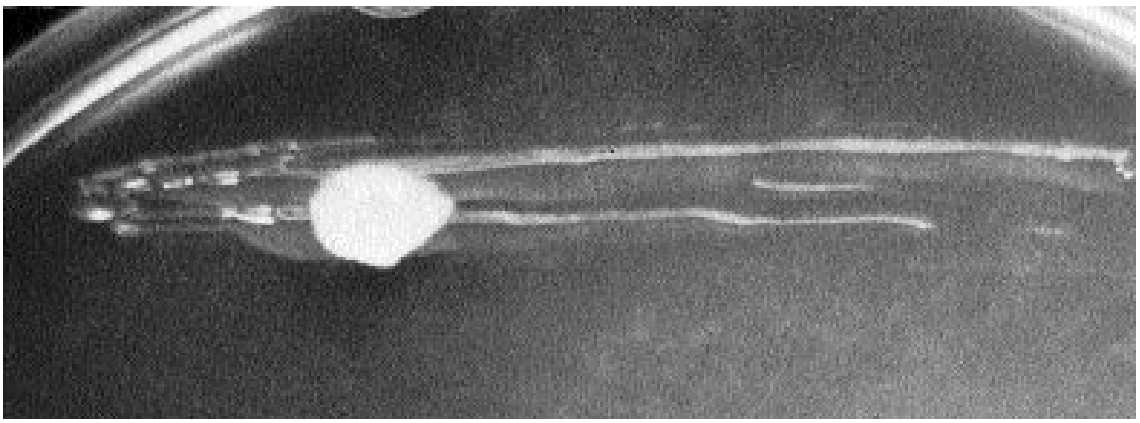

C

Fig. 3. A) *P. avellanae* colony variants obtained after streaking on NSA of 30 day-old colonies (x 2); **B**) single colony of a variant (x 28, left) obtained after incubation at supraoptimal temperature (30°C), compared with a *P. avellanae* colony-type (x 28, right); **C**) occurrence of a *P. avellanae* wild-type colony from colony variants (x2).

strains did not yield variants even on NA with 7% sucrose. After 2, 4 and 6 months of storage of variants on NSA at 25 ± 1°C, it was also possible to obtain 'fresh' variants. After restreaking, these appeared after 3 days. The variants grew well only on NSA and CSGA, whereas very poor growth (small colonies) was observed on NA, NDA, NAG, NB and CVP. No growth at all was observed on KB and GYCA. Sometimes, reversal towards typical *P. avellanae* colonies was observed following restreaking on NSA plates (Fig. 3c). The colony variants did not induce hypersensitivity reaction in tobacco leaves. When stained with Indian ink, the variants showed a halo that might be a capsule. When the lyophilized cultures of variants were revived on NSA after two years we observed the following: (*i*) no growth at all (in most cases); (*ii*) presence of variants alone; (*iii*) presence of typical *P. avellanae* colonies alone; (*iv*) presence of variants and typical colonies.

This study has documented the variability that exists in some characters of *P. avellanae*. Colour and consistency of the colonies varied according to geographic origin. SDS-PAGE and cluster analysis of protein extracts, revealed that strains from northern Italy were different from the others, which showed 90% similarity and clustered in the same group. Also the capacity to liquefy gelatin and the maximum growth temperature indicated that the strains from northern Italy differ from the others. On the other hand, other features were remarkably homogeneous. Thus all strains were negative for ice-nucleation activity as well as for accumulation of poly-β-hydroxybutyrate. They were sensitive to copper sulphate and produced acid from sucrose.

Other characteristics varied according to the number of subcultures. In fact, for the strains isolated in Italy, production of fluorescent pigments on KB and CSGA declined after a number of subcultures on NSA. This behaviour has already been observed for other fluorescent pseudomonads (Palleroni, 1984).

This study clearly indicated that transparent colonies of *P. avellanae* occur in isolation plates. Incubation at supraoptimal growth temperature, the presence of high sucrose content in NA and old colonies on NSA, induce such variants in laboratory. When cultures were more frequently retransferred, the variants did not occur. Production of variants by incubation at supraoptimal growth temperature has already been established for *Erwinia herbicola* (Lohnis) Dye (Chatterjee and Gibbins, 1971). *P. avellanae* variants appeared to be encapsulated and possibly not pathogenic. Interestingly, they were extremely resistant to starvation on dehydrated NSA, being able to revive quickly after 6 months of incubation at 25 ± 1°C. Sometimes, wild-type *P. avellanae* colonies arose spontaneously from the transparent colonies. However, the role of such variants in natural conditions is not yet clear.

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