S.L.J. Tea Sci. **63** (1), 19-27, 1994. *Printed in Sri Lanka.*

ISOLATION AND ENUMERATION OF MICROBIAL INHABITANTS OF TEA PHYLLOPLANE

T. S. Gunasekera

(Tea Research Institute of Sri Lanka, Talawakele, Sri Lanka)

Several microbiological techniques were evaluated to characterize the phyllosphere inhabitants of tea. All had certain deficiencies and it was found that a combination of suitable techniques were required to investigate leaf surface micro-organisms.

Two genera of filamentous fungi, *Cladosporium* and *Pestalotia* and two yeast genera *Cryptococcus* and *Sporobolomyces* were the most numerous fungal and yeast populations isolated. Some fungal spores or hyphae remained attached to the leaf surface after vigorous and prolonged washing, indicating their close association with the leaf. Bacterial colonization was much greater compared with the fungi and yeast on young leaves. Bacteria associated with leaf surface of tea are predominantly gram negative rods and the putative species of *Xanthomonas, Erwinia* and *Pseudomonas* were found to be dominant.

This study indicated that the species composition of the phylloplane micro-flora of tea showed some similarities, with those reported for other plant species.

INTRODUCTION

The aerial surface of plants especially the leaves are colonized by a wide range of micro-organisms including bacteria, yeast and filamentous fungi, some of which are plant pathogens. The occurrence of leaf inhabiting micro-organisms on leaf surfaces is reported in several plant species in both temperate and tropical latitudes.

The studies of phylloplane micro-flora introduced a new concept in disease management: the saprophytes on the leaf surface can act as antagonists to various foliar pathogens, e.g. *Sporobolomyces roseus* as an antagonist to *Septoria nodorum* (Fokkema and Vander Meulen, 1976), *Cochlibolus sativas* (Bashi and Fokkema, 1977) and *Botritis cinerea* (Blakeman and Brodie, 1977); the bacteria *Pseudomonas fluorescence* to *Septoria tritici* (Levy *et al.*, 1992) and *Bacillus subtilis* against *Corticium invisium* (the causal agent of black rot of tea) (Bathker *et al.*, 1993). Possible biological control on the leaf surface has been discussed (Andrews,1992). Further, the isolation of anti-microbial substances from phylloplane yeast (McCormak,1994), production of plant growth promoting auxins by yeast like fungi (Buckley and Pugh, 1971), nitrogen fixation on the leaf surface by free living nitrogen fixing bacteria (Jones, 1970), ice nucleation active bacteria which induce frost damage (Lindow and Cornell, 1984) are some important aspects of leaf surface micro-organisms.

Despite many investigations on other plant species, comparatively little is known about microbial colonization on tea where the component of commercial importance is the leaves. The work described in this paper was designed to investigate the microbial composition of the tea phyllosphere.

MATERIALS AND METHODS

Isolation methods

Two principal approaches were used to investigate the tea leaf surface.

1. **Direct method**: The leaf surface was examined *in situ* by a microscope after decolourising by exposing them to chlorine generated by adding sodium hypochlorite to 1N hydrochloric acid or by the method described by Fernando Watson and Pauitz *et al.* (1993). The cleared and bleached leaves were then stained with Cotton blue in lactophenol and examined for number and type of fungal spores under a light microscope. Leaf clearing and preparation of slides were carried out in a fume cupboard.

2. **Indirect method**: This involved removal of microbial propagules from the leaf surfaces to artificial media and examination after their subsequent growth.

2.1. **Leaf impression technique**: Leaf surfaces were temporarily pressed against a suitable agar media and plates incubated at 22°C for 3 days before colonies were counted.

2.2. **Spore fall technique**: This technique was used to isolate ballistospore producing organisms. Four leaf discs were cut from each leaf and the abaxial surface was attached with petroleum jelly to the lid of a 9 cm diameter Petri dish containing 5% Malt Extract Agar. The plates were exposed to 16 h and they were placed upside down and incubated for a further 48 h before the colonies were counted.

2.3. **Leaf washing technique**: This involved the removal of propagules of micro-organisms from leaf surfaces by employing the cleansing effects of a turbulent liquid and subsequent culturing on agar media. The leaf discs were cut from the leaf and shaken for 15 min. in 20 cm^3 of sterile water containing 0.05% (v/v) Tween 80 using a Griffin flask shaker and the leaf washings were serially diluted in sterile water, the extent of dilution was adjusted to obtain approximately 30-300 colonies when 0.2 cm^3 of the washing was transferred to a suitable growing media.

All the methods and techniques were evaluated for recovery of micro-organisms.

Fungal attachment to the host

Tea clones CY9, DT1 and TRI 2025 were used for the study.

Twenty-five leaf discs, each 1 cm diameter were transferred to 50 ml sterilized water containing 0.05% (v/v) Tween 80 and washed vigorously for 15 min. Leaf discs were washed repetitively (four times) and 0.25 ml of each washings were transferred to separate Petri-dishes containing Potato Dextrose Agar (PDA). The total fungal colonies against *Cladosporium* colonies were recorded. Leaf discs were also cultured on Petri-dishes containing PDA after repetitive washings and different fungal species isolated. The leaf impression technique was also employed by imprinting the leaf serially on PDA plates. Plates were incubated at 22° C for 3 days and total fungal colonies were recorded.

Enumeration and identification of micro-organisms

Only tea flush (the first two leaves and the bud) was used for the sampling process. Leaf washing and spread plate techniques were used for the enumeration. Fifteen leaf discs, each 1 cm in diameter, from the second leaf were shaken for 15 min. in 20 cm^3 water containing 0.05% (v/v) Tween 80. The washings were serially diluted with sterile water before transferring to 9 cm diameter Petri dishes containing Czepek Dox Agar(CDA), Potato Dextrose Agar(PDA), Malt Extract Agar(MEA) or Nutrient Agar (NA). CDA, PDA or MEA was used to isolate fungal and yeast colonies whereas bacteria were isolated on NA. The plates were incubated for 72 h at 22°C in the dark before the colonies were counted. Four samples were taken from July to October, 1994, at approximately monthly intervals.

RESULTS AND DISCUSSION

In the direct microscopic method although the fungal and yeast colonies were observed it was not possible to identify the organisms. Nevertheless it was possible to identify the spores of *Exobasidium vexans* (the causal agent of Blister Blight of tea), *Pestalotia* and *Cladosporium*. The germinating spores of *E. vexans* up to the stage of appressorium formation and some filamentous growth of fungi were noticed. However, this method was found to be too laborious for a quantitative assessment of micro-organisms. Further difficulties were encountered in differentiating a bacterium and a piece of organic detritus shape like bacterium.

Out of the indirect methods, the leaf impression technique provided a quick and simple method of isolating phylloplane micro-flora (Dickinson, 1971). Though the colonies of *Sporobolomyces roseus, Cryptoccocus spp, Cladosporium spp, Pestalotia spp* and some pigmented bacteria were isolated, the number of colonies recovered using this technique were very much less compared to the leaf washing technique (Table 1). The counting of the number of colonies was difficult due to the mixed nature of the population of microbes competing for nutrition and space; further due to their different multiplication rates the slow growing colonies were concealed by fast growing organisms.

TABLE 1 – *Comparison of the leaf impression and the leaf washing technique*

Clone	*Leaf sampled*	*No. of fungal isolates/cm^2 leaf*	
		Leaf impression technique	*Leaf washing technique*
DT 1	1st leaf	13	455
	2nd leaf	21	703
CY 9	1st leaf	09	168
	2nd leaf	20	198

TRI 2024	1st leaf	08	376
	2nd leaf	15	277
TRI 2023	1st leaf	07	277
	2nd leaf	11	416

Spore fall technique was fast and is a reliable method to isolate ballistospore producing fungi; *Sporobolomyces roseus* and *Exobasidium vexans*, were easily isolated using this technique. This method was established by Last (1955) for quantitative studies of *Sporobolomyceteace* on cereal leaves. However, there was no correlation between the number of colonies of *Sporobolomyces* recovered from the spore fall method and the leaf washing technique (Pennycook and Newhook, 1978); thus, ballistospores derived colonies will not be representative of the population of the organism on the leaf surface.

Compared to all other techniques, the leaf washing technique was more efficient, enabling isolation of a greater number and higher recovery of the same species. However, leaf washing technique has certain disadvantages; only a component of the actual population can be satisfactorily isolated while some organisms are difficult to dislodge even after repetitive washing. Further, the degree to which propagules are adsorbed on leaf surfaces from the washing solution is not known. However, addition of a surfactant to the washing media (e.g.Tween) can overcome the above deficiencies to a certain extent.

Fungal attachment to the host

When the leaf surface was washed repetitively most of the superficially growing colonies were isolated. Both tea clones CY 9 and 2025 showed similar trends; and it was possible to remove 70-90% of the fungal population from the first and the second washings. However, some leaf surface fungi can survive on the surface with internal invasion to the host tissues without showing symptoms. Thus, prolonged washing and subsequent culturing of washed leaf tissues would explain any cryptic growth inside the tissue. The fact that *Cladosporium spp.* and *Pestalotia spp.* were isolated from washed leaves, indicated their close association with the leaf surface (Table 2). This experiment was repeated using the leaf impression technique by taking serial imprints which were seen to give similar trends (Table 3). This shows that their occurrence on the leaf surface is not merely by chance but that they are part of the resident flora of the leaf and are able to complete at least part of their life cycle.

TABLE 2 – *Number of Cladosporium colonies out of total fungal colonies at each successive washing*

No. of fungal isolates/cm² leaf

Clone		*TRI 2025*		*CY 9*	
	Leaf sampled	*Total fungal colonies*	*Cladosporium colonies*	*Total fungal colonies*	*Cladosporium colonies*
1st washing	1st leaf	247	85	310	105
	2nd leaf	92	46	33	26
2nd washing	1st leaf	191	26	53	20
	2nd leaf	250	66	20	13
3rd washing	1st leaf	25	12	26	16
	2nd leaf	06	05	18	04
4th washing	1st leaf	17	11	22	17
	2nd leaf	11	05	10	05

TABLE 3 – *Mean fungal isolates cm² on tea leaf surface using leaf impression method (serial imprints)*

Clone	*Leaf sampled*	*1st Imprint*	*2nd Imprint*	*3rd Imprint*	*4th Imprint*
DT 1	1st leaf	19	06	03	02
	2nd leaf	28	14	10	08
CY 9	1st leaf	21	06	04	05
	2nd leaf	26	12	09	06

Micro-organisms of tea phylloplane

Micro-organisms of the flush may be playing some important role in the manufacturing process of tea. In this study emphasis was placed on the micro-flora of the young pluckable shoots, as Blister Blight disease is confined to these shoots. All four samples clearly show that a greater number of bacteria colonize the young shoots compared with fungi and yeast (Fig 1). This was probably due to the maturity of the leaf and the foliar exudates like amino acids and carbohydrates on the leaf surface (Rodger and Blakeman, 1984). Higher number of fungal colonies were isolated from the second leaf as opposed to the first leaf of the shoot. This indicates, that the fungal population tends to increase when the leaf becomes mature. Among the fungal and yeast inhabitants, species of *Cladosporium, Pestalotia, Sporobolomyces, Cryptoccous* were dominant while the species of *Penicillium, Fusarium, Alternaria, Aurobasidum* and Rodotorula appeared occasionally. The fluctuation of two dominant fungal and yeast genera are shown in Fig.2. Most of the bacteria associated with the tea leaf surface were pigmented (Fig.3) and predominantly gram negative rods. Putative species of *Xanthomonas, Erwinia* and *Pseudomonas* are found to be dominant bacterial flora on the tea leaf surface.

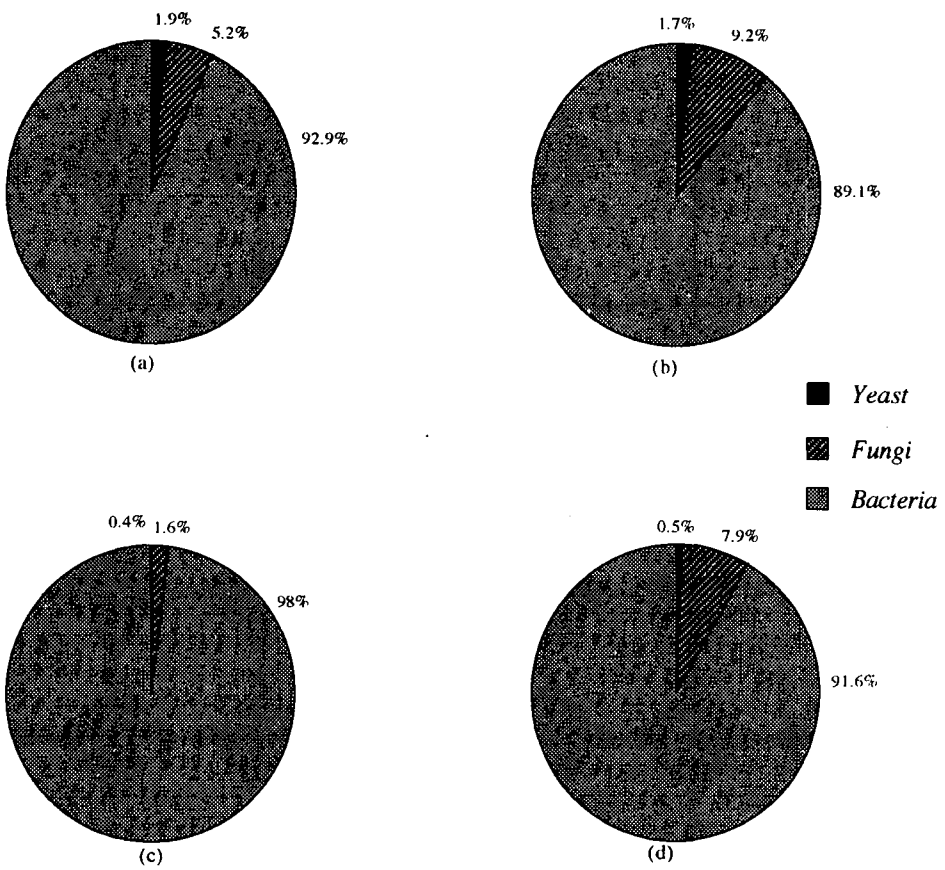

Fig. 1 *(a-d)* – *Trends in microbial colonization on the second leaf of a pluckable shoot. Note: a, b, c, d refer to samples 1, 2, 3 and 4 respectively.*

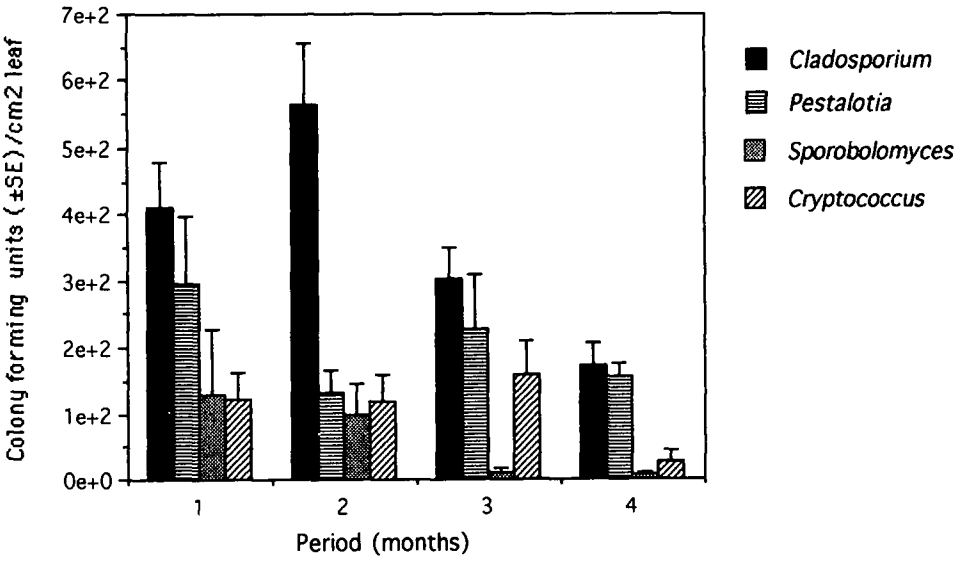

Fig. 2 – *Abundance of dominant genera of fungi and yeast on the second leaf of a pluckable shoot.*

Composition of tea phylloplane micro-organisms shows some similarities to other plant species e.g. European larch (McBride and Hayes, 1977), *Panicum coloratum* (Eicker, 1976), Olive (Ercolani,1978), *Pisum* (Dickinson,1966), Sugar Beet (Thompson *et al.*,1993). Some fluctuations of micro-organisms were observed which was mainly due to environmental fluctuation in the phyllosphere. Although the air is less complex microbiologically, it does exhibit striking diurnal, seasonal and random fluctuation in its physical and chemical characteristics that favour microbial colonization. Therefore micro-organisms living on the leaf surface undergo acute environmental changes and in view of this they show various adaptations (Dickinson, 1985). Pigmentation, thick cell walls, polymorphism, multi-cell spores are some common adaptations among several others. Thus, the leaf becomes an admirable site for some organisms which could withstand extreme climatic conditions.

ACKNOWLEDGMENT

I express my sincere gratitude to late Dr P. V. Arulpragasam, of TRI, Professor P. G. Ayres and Dr N. D. Paul of Lancaster University, UK. for helpful suggestions. I am thankful to Professor S. Widanapathirana of Kelaniya University, Sri Lanka for making arrangements to identify the bacterial isolates.

REFERENCES

ANDREWS, J. H.(1992). Biological control in the phylloshpere. *Annu. Rev. Phytopathol.* **30**, 603-635.

BASHI, E. and FOKKEMA, N. J. (1977). Environmental factors limiting growth of *Sporobolomyces roseus*, an antagonist of *Cochliobolous sativas*, on wheat leaves, *Trans. Br. Mycol.* Soc. **68**, 17-25.

BATHKER, B. K., DUTTA, P., BEGUM, R. and SARMAH, S. R. (1993). *Bacillus subtilis*, a promising bioagent for control of black rot. *Two and a bud.* **40** (2), 50.

BLAKEMAN, J. P and BRODIE, I. D. S. (1977). Competition for nutrients between epiphytic micro-organisms and germination of spores of plant pathogens on beetroot leaves. *Physiol. Plant Pathol.* **10**, 29-42

BUCKLEY, N. G. and PAUGH, G. J. F. (1971). Auxin production by phylloplane fungi, *Nature.* **231**, 332.

DICKINSON, C. H. (1967). Fungal colonization of *Pisum* leaves. *Can. J. Bot.* **45**, 915-926.

DICKINSON, C. H. (1971). Cultural studies of leaf saprophytes. *In Ecology of leaf surface micro-organisms.*(T. F Preece and C. H. DICKINSON, *eds*) Academic press, London. p. 129-137.

DICKINSON, C. H. (1985). Adaptation of micro-organisms to climatic conditions affecting aerial plant surface. In. *Microbiology of the phyllosphere* ed. N. J. Fokkema and J. Van dea Hauel, Academic press, Cambridge. p 77-100

EICKER, A (1976). Non-parasitic micro-flora of the phylloplane and litter of *Panicum coloratum, Tran. Br. Mycol. Soc.* **67**, 275-281.

EROCOANI, G. L. (1978). *Pseudomonas savastanio* and other bacterial colonization of the surface of Olive leaves in the field. *J. Gen. Microbiol.* **109**, 245-257.

FERNANDO, W. G. D., WATSON, A. K. and PAUITZ, T. C. (1993). A simple technique to observe conidial germination on leaf surfaces, *The Mycologist.* **7(4)**, 188-189.

FOKKEMA, N. J. and VANDER DER MEULEN (1976). Antagonism of yeast like phyllosphere fungi against *Septoria nodorum* on wheat leaves. *Neth. J. Plant Pathol.* **82**, 13-16.

JONES, K. (1970). Nitrogen fixation in the phyllosphere of Douglas fir, *Pseudtsuga douglasii, Ann. Rev.* Bot. **26**, 139-152.

LAST, F. T. (1955). Seasonal incidence of *Sporobolomyces* on cereal leaves. *Trans. Br. Mycol. Soc.* **38**, 221-239.

LEVY, E., GOUGH, F. J., BERLIN, K. D., GUIANA, P. W and SMITH, J. T. (1992). Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas* florescence and its antibiotics. *Plant Pathol.* **41**, 335- 341.

LINDOW, S. E. and CORNELL, J. H. (1984). Reduction of frost injury to almond by control of ice nucleation active bacteria. *J. Amer. Soc. Hort. Sci.* **109 (1)**, 48-53.

McCORMACK, J. P., HOWARD, G., WILDMAN and JEFFRIES, P. (1994). Production of antimicrobial compounds by phylloplane inhabiting yeasts and yeast like fungi, *Appl. Environ. Microbiol.* **60(3)**, 927-931.

MCBRIDE, R. P. and HAYES, A. J. (1977). Phyllopane of European larch. *Trans. Br. Mycol. Soc.* **69(1)**, 39-46.

S.L.J. Tea Sci. **63** (1), 19-27, 1994. *Printed in Sri Lanka.*

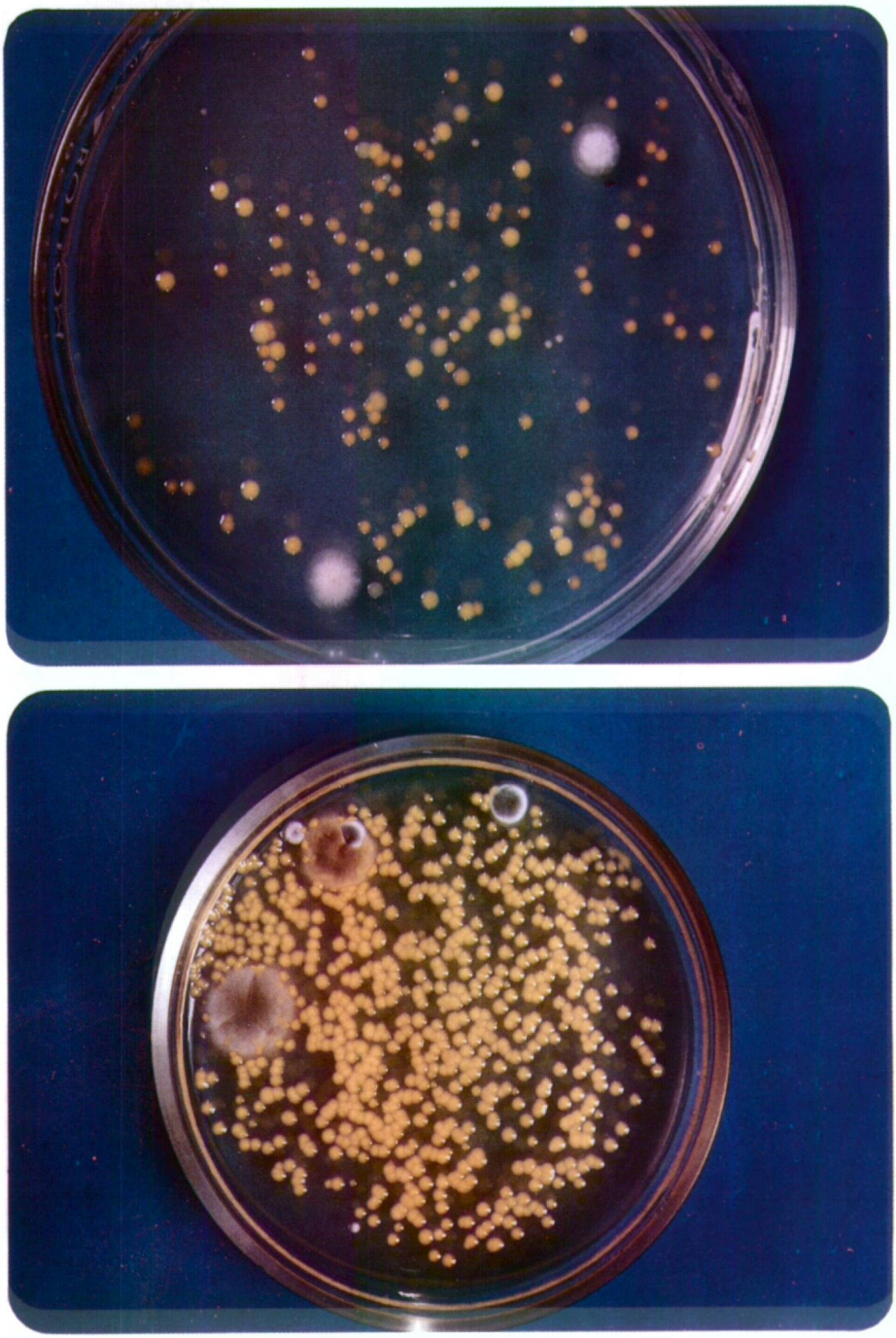

Fig. 3 – *Pigmented bacteria isolated from tea flush, growing on Nutrient Agar.*

PENNYCOOK, S. R. and NEWHOOK, F. H.(1978). Spore fall as a quantitative method in phylloplane studies. *Trans. Br. Mycol. Soc.* **71**, 453-456.

RODGER, G. and BLAKEMAN, J. P. (1984). Microbial colonization and uptake of 14 C label on leaves of sycamore. *Trans. Br. Mycol. Soc.* **82**, 45-52.

THOMPSON, I. P., BAILEY, M. J., FENLON, J. S., FERMOR, T. R., LILLEY, A. K., LYNCH, J. M., McCORMACK, P. J., McQUILKEM, M. P., PURDY, K. J., RAINEY, P. B. and WHIPPS, J. M. (1993). Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (*Beta vulgaris*). *Plant and Soil.* **150 (2)**, 177-191.