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Estimation of *Frankia* growth using Bradford protein and INT reduction activity estimations: application to inoculum standardization

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1. SUMMARY

The growth of *Frankia* spp. strain ORS 020607 in BAP medium was studied by using two methods simultaneously: determination of Bradford protein content and INT (2-(p-iodophenyl-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) reduction activity (IRA). With the latter test, red formazan crystals formed intracellularly were extracted with methanol. Colouration intensity was

2. INTRODUCTION

Frankia is a soil nitrogen-fixing actinomycete whose growth characteristics have been increasingly studied during the last decade. However, because of its filamentous form and very low biomass production in vitro, estimating growth has been difficult. Growth in a liquid medium has been measured through determination of cell den-

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tracellular reduction of tetrazolium salts to red formazan crystals by dehydrogenase enzymes is of great interest because it is simple, rapid and sensitive [4]. The technique has been already used by Akkermans [5] to estimate the metabolic activity of *Frankia* vesicles in relation to nitrogen fixation. To estimate the viability of different structures of *Frankia*, Faure-Raynaud et al. [6] visualized the intracellular formation of INTFormazan resulting from the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) by dehydrogenase enzyme. Extraction of intracellular INTFormazan (INTF) from microbial cells followed by spectrophotometric measurement has also been proposed to quantitatively estimate the viability of microbial populations [7,8]. Prior to this work, INT Reduction Activity (IRA) has never been used to estimate the changes of metabolic activity during *Frankia* growth.

Since in preliminary experiments, IRA occurring in *Frankia* hyphae seemed to reflect differences in hyphal metabolic activity, we attempted to determine whether these variations in INT reduction by metabolically active cells could be used as an indicator of *Frankia* growth. The approach used was to compare IRA with growth as determined by the conventional method of Bradford protein evaluation. Values from IRA and from the Bradford protein method were also compared as a means of standardizing the inoculum used in growth studies.

3. MATERIALS AND METHODS

3.1. Frankia isolate and inoculum preparation

The *Frankia* strain used in this study was strain ORS 020607 isolated from *Casuarina equisetifolia* [9].

Preparation of 8–10 day old inoculum. The actinomycete was first successively cultured three times for 8–10 days. This was necessary to provide an homogenous culture of *Frankia* only composed of young vegetative hyphae. Inocula were obtained by concentrating *Frankia* cultures by centrifugation in sterile 10 ml tubes (10000 × *g* for 10 min at 4°C) using a J2 21 ME Beckman centrifuge (rotor JA-20). The pellets were then washed three times in sterile BAP medium [10]. Finally, colonies were disrupted into small hyphal fragments by forced passages through a 0.8 mm diameter needle. Each inoculum was assayed for its protein content and its IRA.

Preparation of 10, 27, 76 day old inocula. Inocula were obtained by collecting culture samples from a long term culture of *Frankia* at day 10, 27 and 76. Preparation of the inocula was as described above.

3.2. Inoculation, growth conditions and biomass estimations

The suspension of fragmented hyphae was used to inoculate stationary glass tubes (25 × 150 mm) containing 10 ml of BAP medium. After inoculation, the concentration of *Frankia* in the fresh medium was 1.3 μg protein per ml of medium. According to P. Benoist (personal communication) this concentration induced optimal subsequent growth of Frankia. This value was equivalent to 1.3 nmol IRA per ml of medium. Inoculated tubes were incubated at 28°C for 96 days. Throughout this period, estimations of protein and IRA were made at regular intervals from three different tubes.

Subsequently, 3 sets of triplicate tubes containing 10 ml of BAP medium per tube were inoculated with the three inocula obtained at day 10, 27 and 76. Because protein contents varied greatly according to the age of *Frankia*, the amount of inoculum was adjusted so that the final concentration was 1.3 μg protein per ml of medium whatever the age of the culture. 3 additional set of triplicate tubes were inoculated with the same inocula but, as indicated above, the amount of each type of inoculum used was adjusted in such a way that, after inoculation, the final concentration of the inoculum was 1.3 nmol IRA per ml of medium. In each set of triplicate tubes, the time course of Bradford protein content and IRA was followed for 15 days.

3.3. Protein and IRA evaluations

The protein concentration of the homogenized culture was determined with the Bradford procedure [11] using the 'Bio-Rad Protein Assay Kit' (Bio-Rad. U.S.A.). Cell fractions for protein assay

only formed few and small sporangia, and few or no vesicles, structures which are assumed to remain viable for longer periods than hyphae. This could explain the low level of IRA observed after day 15. After day 15 the hyphae became more and more senescent and the production of vesicles or spores was not significant enough to prevent the decrease of IRA. However, at the same time, the Bradford protein content decreased to a lesser extent. This result suggests that, under our experimental conditions, the autolysis of *Frankia* was not significant enough to induce a substantial loss of proteins by leakage of components from the dead cells.

4.2. Relationship between the method of inoculum standardization and the growth curve pattern of Frankia

When the inoculum standardization was based on IRA (1.3 nmol IRA per ml of medium) (Fig. 2), the growth curves derived from the 10, 27 and 76-day old inocula did not significantly differ from each other. This suggests that their inocula contained the same amount of live structures. With the 76-day old inoculum standardized with IRA,

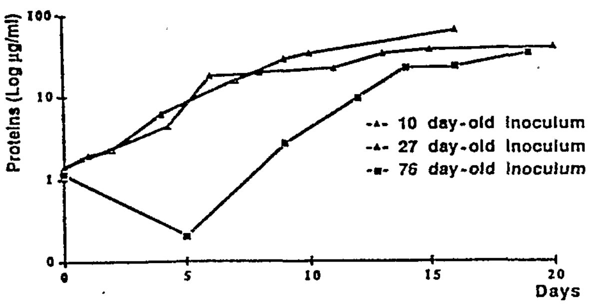

Fig. 3. Growth curves of *Frankia* ORS 020607 using inocula standardized in term of Bradford protein content. In the three cases, the inoculum contained the same amount of Bradford protein (1.3 μg of protein per ml of fresh medium). Each value is the mean of three culture tubes. The standard errors are 4.29, 3.08 and 2.76 for the curves obtained from the 10, 27 and 76-day old inocula respectively.

on Bradford protein (1.3 μg protein per ml of medium), a marked decrease of biomass was observed prior to the active growth phase in the culture inoculated with 76-day old material. The growth was subsequently delayed (Fig. 3). The 10, 27 and 76-day old inocula standardized at 1.3 μg protein per ml of medium had IRA values of 1.3, 0.6 and 0.08 nmol per ml of medium respectively.

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