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Study of two pharmaceutically useful *Psoralea* (Leguminosae) species : influence of inoculation on growth, grain and dry matter yield

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Summary — The germination, vegetative growth, grain yield and dry weight yield were studied in 2 Australian medicinal plants of the genus *Psoralea*: *Psoralea cinerea* and *Psoralea plumosa*. The germination method was shown to be very efficient (germination rate about 90%). The plants were then grown under phytotron conditions, testing nitrogen fertilization compared to inoculation with 3 different *Bradyrhizobium* strains (CB 2063, USDA 3451, USDA 3100). The best grain yields were obtained with strain CB 2063 for *P. cinerea* and with the 2 strains CB 2063 and USDA 3451 for *P. plumosa*. These plants bore abundant fruits and the seeds obtained were able to germinate normally. The growth conditions applied here are thus suited to the 2 species, which will be studied further in a more extensive crop trial (greenhouse or field).

Psoralea cinerea / *Psoralea plumosa* / *Bradyrhizobium* / germination / vegetative growth / yield / furocoumarin

Résumé — Étude de deux espèces de Psoralées (légumineuses) à intérêt pharmaceutique: influence de l'inoculation sur la croissance et les rendements en grain et matière sèche. Nous avons étudié le cycle de développement, depuis la germination jusqu'à la récolte des semences, de deux espèces de Psoralées australiennes à intérêt pharmaceutique : *Psoralea cinerea* et *Psoralea plumosa*. Les semences présentent une double inhibition de germination. La première est d'ordre mécanique (épaisseur du tégument), la seconde a une origine chimique (psoralène contenu dans la semence). Le protocole de germination proposé consiste en un trempage des semences dans de l'eau chaude à 60 °C durant 4 h suivi d'une scarification manuelle. Il s'avère très efficace (taux de germination de l'ordre de 90%). Les plantes ont ensuite été conduites en conditions contrôlées (phytotron) en testant l'effet d'une fertilisation azotée et de l'inoculation de 3 souches de *Bradyrhizobium* étudiées séparément (CB 2063, USDA 3451, USDA 3100). Nous avons suivi le développement de ces plantes par des mesures de hauteur et du nombre de feuilles pendant la période de végétation (figs 1, 2, 3 et 4). Les meilleurs résultats de croissance et de rendement en grain ont été obtenus avec la souche CB 2063 pour *P. cinerea* (fig 5 et tableau I) et avec les 2 souches CB 2063 et USDA 3451 pour *P. plumosa* (fig 6 et tableau II). Les plantes des traitements correspondants ont fructifié abondamment et les semences récoltées sont aptes à germer normalement. Les conditions de végétation appliquées conviennent donc parfaitement aux 2 espèces étudiées qui se prêteront à l'avenir à un essai de culture sur de plus grandes surfaces (serre ou plein champ).

Psoralea cinerea / *Psoralea plumosa* / furocoumarines / *Bradyrhizobium* / germination / croissance végétative / rendement

INTRODUCTION

The genus *Psoralea* (Leguminosae) is rare in Europe, with a single species in the mediterranean area (*P. bituminosa*), but is well represented in the arid zones of South Africa, North America and Australia (Allen and Allen,

1981). Apart from their value in pasture improvement in hot regions (Dorbec, 1985), these plants are of some interest to the pharmaceutical industry since they produce active substances (furocoumarins) used in cosmetic products, and increasingly, in dermatology.

* Correspondence and reprints

At present, these compounds are essentially obtained from bergamot oil (Bourgaud *et al*, 1989) since their chemical synthesis is prohibitively expensive. However, in view of the low levels of bergamot (*Citrus bergamia*), production it is advisable to turn to other richer plant resources to meet the increasing requirements of the pharmaceutical industry.

A recent publication (Innocenti *et al*, 1984) has shown the value of Australian species of *Psoralea* which synthesize appreciable contents of furocoumarins in the seeds (depending on the species, the seeds contain 1 000-8 000 ppm total furocoumarins per g dry weight). This finding led us to turn our attention to this group of plants and notably to two species, *P cinerea* and *P plumosa* (being the richest in furocoumarins among those analysed by Innocenti *et al*, 1984) with a view to subsequent cultivation in greenhouses or in the field. Since nothing is known about furocoumarins concentration in the other parts of the plants (especially vegetative organs), we decided to study the whole plants in order to determine later the most interesting tissues for production of furocoumarins.

These are two species originating from the semi-arid regions of Western Australia (350 mm annual rainfall, mean maximum temperature 35 °C, mean minimum temperature 11 °C) and growing best in sandy soils (Beadle, 1964; Torrey and Gray, 1969, Keighery and Dixon, 1981). Since they are legumes they establish symbiosis with *Rhizobium* of the "cowpea" type (slow growing *Rhizobium*, now classed as *Bradyrhizobium*) and thus contribute to enriching the soils on which they grow (Beadle, 1964). However, there have been few studies of nodulation and crossed inoculation experiments in *Psoralea* species (Appleman and Sears, 1942), nor is there any available information on the growing cycle of these plants. These plants also present problems in germination due to the occurrence of a double inhibition (Baskin *et al*, 1967): mechanical inhibition: the seeds possess a very thick tegument preventing their rehydration; chemical inhibition: the pericarp contains a substance that inhibits germination. This substance is a furocoumarin (psoralen) which is 1 of the medically interesting metabolites.

In this initial study, we first sought to determine the appropriate technique for germination of the 2 plants. We then studied the vegetative growth cycle under controlled conditions, with particular emphasis on the flowering-maturation phase. With a view to later utilisation and improvement, the major objective was to understand the factors involved in determining grain yield. The third

stage was to determine the growth conditions giving optimal biomass and grain yields. To this end, we considered it essential to test the importance of the *Psoralea-cowpea Rhizobium* symbiosis to the growth of these two chosen species, using different bacterial strains. Nitrogen fertilization and cowpea *Rhizobium* inoculation were thus compared in order to develop a cultivation method adapted to good vegetative growth and allowing the production of effective biomass and grain yield.

MATERIALS AND METHODS

Germination test

The seeds (*P cinerea*, *P plumosa*), supplied by the Perth botanical gardens, were placed in warm water (60 °C) for 4 h and their tegument was then nicked, taking care not to injure the cotyledons (the seeds are exalbuminous). The seeds treated in this way were placed in moist filter at 25 °C in the dark.

Experimental methods

Cultivation of the plants

After germination, the seedlings were transferred to vermiculite moistened with the nutrient medium described by Wood *et al* (1983) until the 2-leaf stage. They were then replanted into individual pots (10x10x11 cm) containing 130 g of a sand-gravel mixture in the bottom and 600 g sandy earth on top. The pots were placed in a growth chamber under the following conditions: 15 h of illumination at 25 °C, 9 h of darkness at 18 °C, 70% r h. The illumination was approximately 600 $\mu\text{E m}^{-2} \text{s}^{-1}$. The soil was kept at 2/3 of the water holding capacity.

Selection and cultivation of *Rhizobium*

Among the *Rhizobium* cultures available to us we chose 3 strains for their ability to induce nodulation of *Psoralea* plants: CB 2 063 isolated from *Psoralea cinerea* in Australia (Brockwell, personal communication);

USDA 3 100: isolated from *Psoralea psoraloides* in the USA (Keyser and Griffin, 1987);

USDA 3 451: isolated from *Macrotyloma africanum* in Africa. This strain is known to be able to nodulate a large number of tropical leguminous species (Keyser and Griffin, 1987).

The selected strains were cultivated in liquid yeast extract-mannitol broth medium (0.4 g/l) (Vincent, 1970) and were inoculated at a rate of 10^9 bacteria per plant. The roots were examined at the end of the experiment to determine the presence or absence of nodules.

Experimental design

Five treatments, each in 10 replicates, were applied to each of the 2 studied species: 1) control: plants

without fertilizer and not inoculated; 2) nitrogen: plants fertilized with ammonium nitrate at a rate of 50 units of nitrogen per ha; 3) CB 2 063: plants inoculated with bacteria of strain CB 2 063; 4) USDA 3 451: plants inoculated with bacteria of strain USDA 3 451; 5) USDA 3 100: plants inoculated with bacteria of strain USDA 3 100.

The relatively low nitrogen dose of 50 units \cdot ha⁻¹ was adopted since *Psoralea* species grow on poor soils. Knowing that the first nodules have been observed at the 3-4 leaf stage in the plants of the same genus (Beadle, 1964), the bacterial inoculum and nitrogen application were given at the 4-leaf stage.

Growth monitoring-Estimation of yield

Growth monitoring

Biometric measurements were undertaken twice weekly: plant height from the stem base to the terminal bud; total number of leaves per plant. The emergence of flower buds, the start of flowering and the maturation stage were also noted for each species and each treatment.

Estimation of yield

The seeds harvested as they matured were placed in an incubator at 25 °C (a temperature not affecting the germinating potential of the seeds so that they could be utilized later) for 2 weeks in order to obtain homogeneous moisture contents. At the end of the experiment the different plant organs were separated (roots, stems, leaves, immature fruits) and dried in an oven at 35 °C for 2 weeks to obtain the dry material yield of these organs per species and per treatment (this relatively low temperature does not affect any furocoumarins that may be present so that they can be assayed in a future study).

Concerning yield parameters, we used the following formulation:

$$\text{yield/plant} = (\text{number of inflorescences/plant}) \times (\text{number of fruits/inflorescence}) \times (\text{number of seeds/fruit}) \times (1\ 000 \text{ seed weight}) / 1\ 000$$

As there is always only 1 seed per fruit, we obtain: (number of fruits/inflorescence) = number of seeds/inflorescence. The parameters: yield/plant, number of inflorescence per plant, 1 000 seed weight were measured at the end of the vegetation period. The number of seeds/inflorescence was not measured but calculated.

Statistical analysis

The twice weekly biometric data were interpreted by analysis of variance and the Newman-Keuls test for comparison of means in order to notice if there were significant differences due to inoculation/fertilization between the treatments.

During harvest, we determined standard deviation for seed, leaf, stem and root weight within each treatment, but not for immature fruits.

RESULTS

The observations were made over a period of 130 d in the case of *P. cinerea* (equivalent to 2 900 degree-days when summing the positive temperatures) and 140 d in the case of *P. plumosa* (corresponding to 3 090 degree-days). The results for germination method are presented first, followed by those relating to the growth of aerial and reproductive organs, with particular attention paid to the role of inoculation.

Germination

Using the method described above, applied to batches of 50 seeds per species, germination rates of 90% were obtained with *P. cinerea* and 96% with *P. plumosa* after 48 h in an incubator. The only seeds which did not germinate showed cotyledons or embryos damaged by insect attack. In parallel with this, the possibility of germinating seed batches of both species without warm water treatment or scarification was tested. The filter paper then showed brown haloes around the seeds, effectively demonstrating the diffusion of a water-soluble compound that is probably psoralen (Baskin *et al.*, 1967). However, no germination was found after 15 d. These 2 batches of 20 seeds were then scarified by hand and germination occurred within 24 h (95%).

The method proposed by Baskin *et al.* (1967) thus proved to be very efficient with both tested *Psoralea* species and the need for 2 interventions: steeping in warm water and scarification, can be confirmed.

As a complement to these observations, the possible existence of dormancy other than the said chemical and mechanical dormancy was examined (comparable to the thermodependent physiological dormancy of wheat, for example). The seeds collected after the first vegetative season of our plants were used for this purpose. Fifty seeds per species were subjected to the germination procedure less than 1 month after harvesting and, after warm water treatment and scarification, a germination rate of 95% was recorded. Consequently, the seeds obtained under our conditions are able to germinate and there seems to be no other type of inhibition of germination (physiological dormancy).

Vegetative growth

Height

Under our conditions the plants reached a maximal height of about 60 cm after 130 d of

growth in the case of *P. cinerea* (fig 1) and 30 cm after 140 d in the case of *P. plumosa* (fig 2). The effect of the fertilization-inoculation factor on the height of the 2 species was significant from the 5th day after the treatment until the end of the experiment. In *P. cinerea*, the best results for growth in height were obtained with strain CB 2 063 from the 19th day. In contrast, in *P. plumosa* these strains induced only a moderate growth curve (same results with strain USDA 3 451) ending in a plateau corresponding to the reproductive period of this species. *P. plumosa* inoculated with these 2 strains developed a lot of reproductive organs which, on the other hand, induced slow growth in height compared with control. Plants inoculated with strain USDA 3 100 showed a curve comparable to that of the controls.

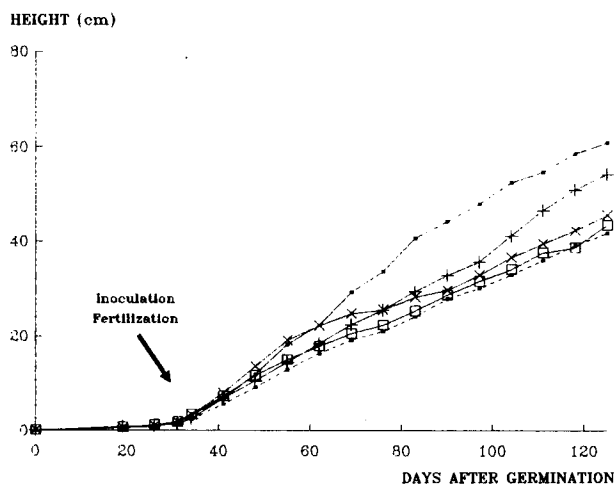


Fig. 1. Effects of treatments (bacterial inoculations or nitrogen fertilization) on growth in height (cm) of *Psoralea cinerea* during the days after germination. —■— CB 2 083; —▲— USDA 3 451; —□— control; —○— USDA 3 100; —×— nitrogen.

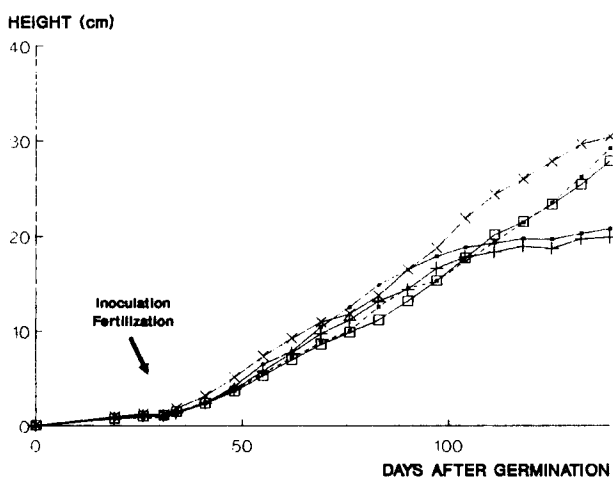


Fig. 2. Effects of treatments (bacterial inoculations or nitrogen fertilization) on growth in height (cm) of *Psoralea plumosa* during the days after germination. —■— CB 2 083; —▲— USDA 3 451; —□— control; —○— USDA 3 100; —×— nitrogen.

Total number of leaves

Both species have trefoil leaves, the number of which reaches about 20 for *P. cinerea* and over 25 in the case of *P. plumosa*. One leaf needs 6 d to develop completely for *P. cinerea* (134 degree-days) and 7 d in case of *P. plumosa* (157 degree-days). The effect of fertilization-inoculation was significant from the 23rd day for *P. cinerea* but only from the 45th day for *P. plumosa*. In *P. cinerea* (fig 3), only treatment with CB 2 063 led to a large number of leaves (the 2 decreases observed were due to accidental drying). In *P. plumosa* (fig 4) the 2 treatments with CB 2 063 and USDA 3 451 led to a large number of leaves, whereas the plants fertilized with nitrogen had only an average number of leaves. The controls and plants treated with USDA 3 100 showed a constant number of leaves since the plants shed

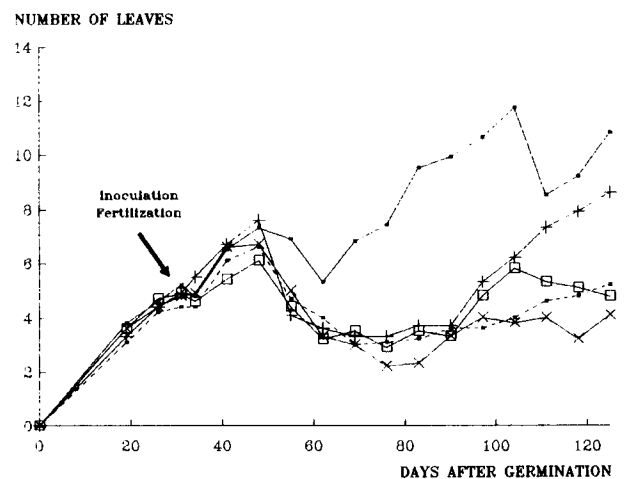


Fig. 3. Effects of treatments (bacterial inoculations or nitrogen fertilization) on total number of leaves of *Psoralea cinerea* during the days after germination. —■— CB 2 083; —▲— USDA 3 451; —□— control; —○— USDA 3 100; —×— nitrogen.

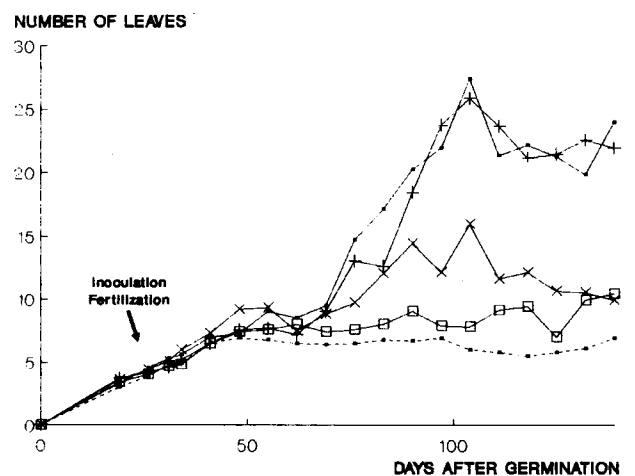


Fig. 4. Effects of treatments (bacterial inoculations or nitrogen fertilization) on total number of leaves of *Psoralea plumosa* during the days after germination. —■— CB 2 083; —▲— USDA 3 451; —□— control; —○— USDA 3 100; —×— nitrogen.

their old leaves as they were replaced by new. In the case of *P. cinerea* treated with USDA 3 451, an increase in the total number of leaves was observed from the 90th day.

Flowering

The flower buds in both species appear on long floral peduncles in the leaf axils from the 6th or 7th leaf. The inflorescences are spikes in which the lateral buds each give rise to 3 flowers. There are 10-30 flowers per inflorescence in *P. cinerea* and 10-15 in *P. plumosa*.

The flower buds appeared around the 40th day after germination with all treatments of *P. cinerea* (about 900 degree-days) and around the 60th day (about 1 340 degree-days) with all treatments of *P. plumosa*. Flowering was basifugal along the stem, with staging of maturation from the base to the apex of each plant. In both species, the flowering-fruiting period lasted for a period of \approx 2.5 months, a flower remained 15 d (335 degree-days) on the plant, and the maturation lasted 20 d (450 degree-days). Since the plants had been kept within a phytotron throughout their growth cycle, fruiting must have resulted from self-pollination of the flowers.

Grain and dry matter yields

Dry matter yield

In addition to the biomasses of the leaves, stems and roots, interest also centered in the number of immature fruits since they are often the parts richest in furocoumarins (Innocenti, personal communication). The results were very clear for *P. cinerea* (fig 5). The best yields were obtained with strain CB 2 063, closely followed by USDA 3 451 treatment. With all treatments the leaf biomass was equal to that of the roots, whereas the stems amounted to at least twice the leaf biomass. The importance of the stems and the low leaf biomass are related to the erect habit of this plant.

The results were less clear with *P. plumosa* (fig 6). Ignoring the values relative to the roots, the greatest aerial biomasses (leaves plus stems) were obtained with strain USDA 3 451 in relation to the leaves and immature fruits, and with nitrogen in relation to stems. However, the aerial biomass remained low (5 g/10 plants for stems with nitrogen treatment) compared with that observed with *P. cinerea* (over 10 g/10 plants for stems with CB 2 063 treatment). *P. plumosa*

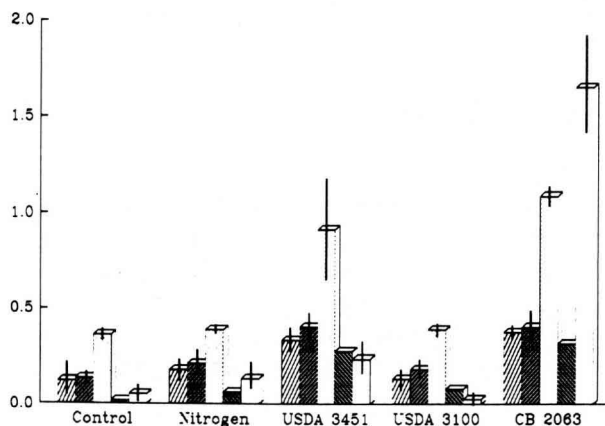


Fig. 5. Effects of treatments (bacterial inoculations or nitrogen fertilization) on dry matter yields (g/plant) in *Psoralea cinerea*. \diagup leaves; \times roots; --- stems; |||| immature fruits; \square seeds. The vertical bars are standard deviations of the means.

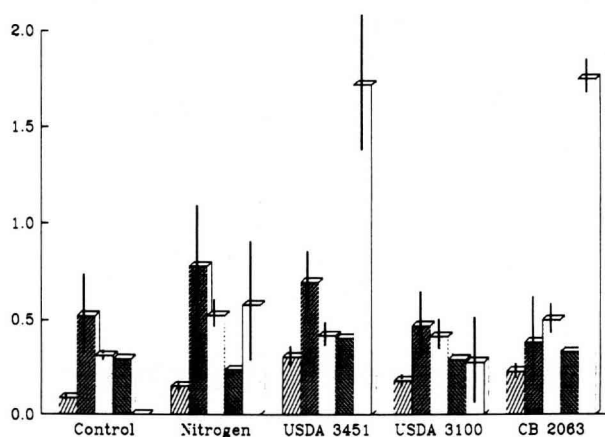


Fig. 6. Effects of treatments (bacterial inoculations or nitrogen fertilization) on dry matter yields (g/plant) in *Psoralea plumosa*. \diagup leaves; \times roots; --- stems; |||| immature fruits; \square seeds. The vertical bars are standard deviations of the means.

attained a lower height than the other species and exhibited a more bushy habit.

Grain yield

The seeds were harvested during 64 d in the case of *P. cinerea* and 72 d in the case of *P. plumosa*. The grain yield histograms (fig 5 for *P. cinerea* and fig 6 for *P. plumosa*) confirm the results reported for vegetative growth. The most effective treatments were: strain CB 2 063: 16.5 g/10 *P. cinerea* plants; 17.5 g/10 *P. plumosa* plants; strain USDA 3 451: 17.2 g/10 *P. plumosa* plants. This strain did not give high yields with *P. cinerea*.

Nitrogen treatments provided an average production of seeds by *P. plumosa* (6.3 g/10 plants) but only a low yield with *P. cinerea*

(1.3 g/10 plants). As with vegetative growth, the controls and plants treated with USDA 3 100 gave only low seed yields.

Yield parameters

Tables I and II show yield parameters for *P cinerea* and *P plumosa*. For *P cinerea*, all the parameters vary strongly according to the treatments. But the number of inflorescences per plant is the most sensitive parameter to nitrogen nutrition, followed by number of seeds per inflorescence and, lastly, 1 000 seed yield.

For *P plumosa*, we can notice considerable variations with the number of inflorescence per plant depending on the type of fertilization/inoculation. On the contrary, the number of seeds per inflorescence and 1 000 seed weight show little differences from 1 treatment to another. The most important difference between the 2 species is observed with 1 000 seed weight parameter.

Presence of root nodules

The roots systems were observed at the end of the vegetative cycle. Both species inoculated with strain CB 2 063 showed nodules with a

maximum diameter of 3 mm. *P plumosa* inoculated with strain USDA 3 451 formed nodules as large as 7 mm. In all these cases the nodules were distributed uniformly over the root mass. In contrast, the nodules arising on *P cinerea* inoculated with USDA 3 451 had the same appearance as those formed by strain CB 2 063 but were located only at the periphery of the root system, corresponding to young roots. Plants inoculated with strain USDA 3 100, those fertilized with nitrogen and the controls did not form nodules.

DISCUSSION

Developmental cycle

The proposed germination method proved to be very effective with a high level of germination (90%) within a short time (24-48 h). Two major advantages derive from this: economy in seeds, which is valuable since the seeds come from Australia; the production of synchronous groups of seedlings for growth experiment. The drawback lies, however, in the laborious scarification of the seeds, which makes the method unsuitable for cultivation on a larger scale (greenhouse or field). Chemical scarification tests are being considered for the future.

Table I. Yield parameters for *P cinerea* growing with different inoculation/fertilization treatments.

TREATMENTS	yield (g/plant)	inflorescence number per plant	seed number per inflorescence	1000 grain weight
CB 2063	1.65	14.3	28.1	4.11
USDA 3451	0.24	10.2	9.5	2.44
USDA 3100	0.03	1.7	8.6	1.77
50 uN/ha	1.33	3.8	17.3	2.03
CONTROL	0.05	1.7	15.2	2.05

Table II. Yield parameters for *P plumosa* growing with different inoculation/fertilization treatments.

TREATMENTS	yield (g/plant)	inflorescence number per plant	seed number per inflorescence	1000 grain weight
CB 2063	1.75	10.9	14.3	11.26
USDA 3451	1.72	10.1	15.4	11.08
USDA 3100	0.28	2.8	10.0	9.89
50 uN/ha	0.63	4.0	15.4	10.21
CONTROL	0	0	ND	ND

With regard to the developmental cycle, the conditions chosen here allowed the cycle to be completed with highly satisfactory fruiting and maturation leading to seeds with a 99% germination capacity. These annual plants thus have a growth period and require growth conditions allowing greenhouse or field cultivation trials under suitable climatic conditions to be seriously considered at a later stage. In our experimentation the temperature summations recorded correspond to mediterranean climate.

Comparison between nitrogen fertilization and inoculation

The application of a nitrogen dose equivalent to 50 U/ha had only a mediocre effect on plant growth and yields. Indeed, with *P. cinerea* the vegetative growth parameters (height and total number of leaves) as well as the grain and dry matter yields were low. *P. plumosa* responded no better to the application of nitrogen and only the root and stem yields were high. In effect, better growth in height was observed in plants fertilized with nitrogen, whereas the plants treated with USDA 3 451 and CB 2 063 ceased their linear growth at the start of the main fruiting period. The nitrogen dose of 50 U/ha seems to have been too small to ensure growth equivalent to that obtained with effective inoculations, which indicates the nitrogen requirements in these plants.

The best growth and yield results with *P. cinerea* were obtained with strain CB 2 063. This strain had been isolated from a plant of this species in Australia so that it has good specificity for *P. cinerea*. In parallel, CB 2 063 also provided good growth and fruiting of *P. plumosa* so that one and the same strain can therefore establish symbiosis with several species of the genus *Psoralea*. With *P. plumosa*, strain USDA 3 451 gave growth and yield results comparable to those obtained with strain CB 2 063. Since this strain had been isolated from an African legume (*Macrotyloma*), it can induce nodules in plants not belonging to even the same genus. This strain thus has low specificity.

With regard to the results obtained with the combination of USDA 3 451 and *P. cinerea*, the location of the nodules on the young roots at the periphery of the root system has already been emphasized together with their resemblance to the nodules induced by strain CB 2 063. These 2 observations and the late appearance of the inoculation effect with strain USDA 3 451 suggest that this treatment replicate had been contaminated with bacteria of strain CB 2 063

towards the end of the experiment. Strain USDA 3 451 is, in fact, unable to establish symbiosis with *P. cinerea* under our experimental conditions.

Even though it had been isolated from an American species of *Psoralea*, strain USDA 3 100 did not form nodules with either of the 2 studied *Psoralea* species. The growth and yield results with this treatment applied to the 2 species were comparable in all respects with those obtained in the 2 controls. With regard to the controls, it should also be noted that neither of the 2 plants species exhibited root nodules: the *Rhizobium* present in the soil in the natural state was unable to establish symbiosis with the *Psoralea* species.

CONCLUSION

Despite the small number of strains tested (3) and despite the absence of bibliographic references concerning the growth, fruiting and nodulation of *Psoralea*, satisfactory growth conditions were determined for the controlled cultivation of the 2 studied species: *P. cinerea* and *P. plumosa*. Good vegetative growth as well as optimal seed yields were obtained with strain CB 2 063 associated with *P. cinerea* and with strains CB 2 063 and USDA 3 451 in the case of *P. plumosa*. A later study should specify the localization and quantity of furocoumarins present in each species in order to determine the respective values of each organ of these plants. This information derived from the present results suggests that both these species can be cultivated on a larger scale (greenhouse or field) in a suitable climate.

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