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Commercial inoculant products for subclover that are marketed in the Pacific Northwest were evaluated in field trials (supplemented by appropriate laboratory analysis) at four sites in southwest Oregon. The materials tested were Nitragin (Milwaukee, WI) and Northrup-King (Minneapolis, MN) peat-based inocula, and Celpril (Manteca, CA) inoculated, lime-pelleted subclover seed. Duplicate plots were included for the peat inocula at seeding rates equivalent to 136 kg seed/ha and the seed was inoculated according to the manufacturer's instructions and at twice the recommended dosage. The Celpril seed was tested at 196, 98, 49, and 25 kg seed/ha. (duplicate plots) and subclover variety Mt. Baker was used for all field trials. In plant infection assays, the Nitragin produce contained $3.7 \times 10^5$ rhizobia/gram peat which were moderately effective while the Northrup-King peat yielded $3.0 \times 10^4$

rhizobia/gram peat which were ineffective. The Celpril seed contained 77.3 rhizobia seed which were highly effective. Laboratory analysis showed that for the peat-based products the probability of ineffective nodulation was high. The field plots were established in October, 1978. The plants germinated and were then exposed to an unusually harsh winter which included the coldest average January temperature ever recorded for Oregon. Most of the plants inoculated with Nitragin and Northrup-King survived the winter and by March, 1979, had developed 12-14 true leaves and an immature, but well nodulated root system. The Nitragin-inoculated plants generally showed higher yields than Northrup-King-inoculated plants, most of the difference between them generally were statistically significant. In the Celpril plots, only a few plants at the higher seeding rates survived the winter. Those which survived showed vigorous growth and dry weights were generally significantly greater than those for the peat-based inoculated seeds. The results indicate that for winter legumes, a commercial product must contain a sufficient number of effective rhizobia, which are able to tolerate wet, cold soil conditions. Above all, no inoculant should contain rhizobia which ineffectively nodulate subclover. Celpril proved to be the superior product. This may be because pelleted products inoculated with single effective rhizobia are superior to multistrain peat-based inocula.

# THE EFFECTS OF COMMERCIAL *RHIZOBIUM* INOCULANTS ON THE ESTABLISHMENT OF *TRIFOLIUM SUBTERRANEUM* IN SOUTHWEST OREGON SOILS

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## INTRODUCTION

Legumes such as clovers and alfalfa have been considered significant factors in improvement and maintenance of soil fertility (Moreley, 1961; Cameron, 1959). Since "fertile soil ranks first among any nation's resources," (Allen, 1973) forage legumes take on added signfiicance especially when used to improve infertile soils. With fertile soils there is economic benefit. This is evident in the desire of sheep ranchers in southwest Oregon to improve and expand their grazing capabilities by sowing previously cleared hill sides with *Trifolium subterraneum*.

Rampton (1945) related the suitability of subterranean clover for pasture in Oregon west of the Cascades. But over the years, subclover's potential for producing fine pasture for sheep grazing and improving and maintaining soil fertility has not been fully exploited. One reason for this apparent failure lies in a statement by Rampton regarding seed inoculation, viz., "subclover develops root nodules with the same nodule bacteria that inoculate white, red, crimson, and alsike clovers" (Rampton, 1945). However, it has been demonstrated "that the association between plant species and rhizobial strain is highly specific" (Allen, 1973). Nodulation does not

mean that dinitrogen fixation will occur, i.e., display effectiveness.

Mixing rhizobial strains which are not effective for the same cultivar may prove to be detrimental. One strain which is effective for a cultivar other than the one whose seeds are inoculated may out-compete the effective strain for infection sites on the seedling and thus cause ineffective nodulation.

There has been and still is the problem of inoculating subclover seed with effective rhizobial strains. Apparently the commercial inocula available to farmers and ranchers have failed and subclover pasture has not been developed as it could. With the renewed interest in establishing subclover pasture, more significance is placed on the effectiveness of the commercial inocula which are sold to producers who are planting subclover. It is the purpose of this research, using field trials and laboratory analysis, to determine if the commercial inocula is adequate or not for the development and persistance of subclover pasture.

# 2. LITERATURE REVIEW

## 2.1 The Suitability and Benefits of Subterranean Clover for Forage in Oregon West of the Cascades

*Trifolium subterraneum* is an annual, self-fertilizing, winter-growing forage crop. It is a long-day plant and requires vernalization (Morely, 1961). Growth during the winter is slow but accelerates dramatically during the spring. Seeds mature by mid-summer and the plant dies. Since 'subclover' is adapted to relatively warm, moist winters and dry summers and can survive temperatures as low as $-12^{o}C$, the plant appears to be well suited to the area of Oregon west of the Cascades (Rampton, 1945). In Oregon there are approximately 640,000 acres of alfalfa and clover hay, 56,000 acres of legumes used for seed, and about 500,000 acres of grass and clover pastures (Hagedorn, 1978a). For the particular conditions of Oregon two cultivars of subclover appear to be especially suitable: (i) Mt. Barker, and (ii) Tallarook (Rampton, 1945).

It has been demonstrated that subclover can improve soil fertility and structure and minimize erosion (Cameron, 1959a, 1959b). Many ranchers in Oregon want to exploit these characteristics of subclover, since many of the soils sown to subclover for forage are located on hill slopes, valley terraces, mountainous uplands, and large

areas which had previously been clear-cut. Generally, these soils are shallow and well drained, have a moderately fine texture, and display low organic matter content and low base saturation (Hagedorn, 1978b).

Biological fixation of dinitrogen through the symbiosis between subclover and strains of Rhizobia trifolii is the most important factor of fertility in the establishment of a grass/clover pasture (Moreley, 1961). Moreley (1961) reported a turnover of approximately 9-18 kg of nitrogen per hectare per year in a productive grass/clover pasture which is in nitrogen equilibrium. Other investigators have found that the turnover rate of fixed nitrogen ranges between 42-84 kg of nitrogen per hectare per year (Allen, 1973). Simpson (1965) demonstrated in greenhouse pot experiments that subclover transfers fixed nitrogen to grasses which are grown in association with it. He showed that transferance of fixed nitrogen was significant but occured only at senescence; thus, during the late spring to summer months when subclover sheds its nodules and dies, there is considerable transferance of fixed nitrogen to the associated grasses.

## 2.2 Establishment of Subclover and Commercial Rhizobia Inoculants

For the successful establishment of clover in prepared seed beds and on open range, the soil must have certain physical characteristics suitable for plant growth, sufficient nutrients, and if available nitrogen is in low concentration, an effective rhizobial inoculant (Moreley, 1961). An effective rhizobial inoculant, defined as one which nodulates legumes and demonstrates dinitrogen fixation, is also necessary when there are naturalized ineffective rhizobia which could noculate but not fix dinitrogen (Ireland and Vincent, 1968).

The added factor of competition for nodulation by ineffective strains of Rhizobia makes the probability of establishing a subclover pasture more difficult. The necessity of effective strains or Rhizobia which can compete against naturalized ineffective strains in nodulation of the host legume is a primary factor in establishment of any legume. Of course the application of fixed nitrogen would alleviate the problem of having to deal with a rhizobial inoculant, but such recourse is not energy conserving and is becoming subsequently more of an economic burden (Date, 1970). For the farmer seeking a more effective way to conserve energy and money, commercial rhizobial inoculants are available; however, commercial inoculants are historically

unreliable in comparison with commercial fertilizers (Burton, 1967).

What the producer needs is a high quality inoculum. Date (1970) stated that "high quality is the end result of a rather rigorous program involving selection of rhizobial strains, growth of rhizobia in pure culture fermentation and impregnation of a carrier material such as finely ground peat, with a rhizobial broth to meet minimum standards in respect of numbers and freedom from contamination by other organisms."

## 2.3 Rhizobium Strain Selection

An inoculum strain is selected primarily for commercial distribution if it demonstrates sound effectiveness (Date, 1970). Ideally such a strain would at least enable the inoculated plant to develop as much dry-matter and as high a percent nitrogen as would the application of fixed nitrogen. In addition to demonstrating sufficient dinitrogen fixation, strains displaying a wide spectrum of specificity, i.e., they are effective on many different plant species and cultivars, are thought by some investigators such as Burton (1967) to be "far more practical and desirable than those which can be used on only one species or variety of plant".

Species of the genus Trifolium mannifest cross-infection, i.e., a single strain of R. trifolii may infect more than one species of clover. Burton (1965) demonstrated that some strains of R. trifolii would effectively nodulate up to 3 species of clover. However, in experiments where several species of clover were inoculated with multiple strains of R. trifolii, the majority of the R. trifolii strains were effective on only one or two species of clover.

The problem of ineffective inoculum strains out-competing effective strains was demonstrated by Burton and Allen (1949). They showed that a strain of R. trifolii which was effective for white clover but not for crimson clover would out-compete a strain of R. trifolii which was only effective for crimson clover for infection sites on crimson clover seedlings. The specificity of R. trifolii is not just between species. Brockwell, et al. (1968) demonstrated that the strain of R. trifolii, TA1, was effective on several cultivars of subclover but not for Woogenellup, a commonly used subclover cultivar. In fact many investigators have demonstrated that ineffective rhizobia may impede nodulation by effective strains (Burton and Allen, 1949; Means, et al., 1961).

In the U.S., farmers usually find that the commercial inocula they buy is not for a single host but for a variety of species. Does this mean that there is a single,

multiple-host strain of R. trifolii which has been proven effective for several species or cultivars within a species? Or does it mean that the inoculum is comprised of several strains each being effective for a few cultivars? Burton (1967) stated that "in preparing a multiple host inoculant, no rhizobial strain should be included which ineffectively nodulates any of the legumes for which the inoculant is prepared." This is a rule which must hold if a multiple host inoculant is to benefit the farmer.

Multistrain inoculants are prepared primarily to simplify distribution, offset possible undesirable mutations, increase the range of host plants, and to protect against bacteriophage attack. But competition between the strains in peat and in the soil environment increases the unpredictability of the inoculum. One strain, X, may so dominate another, Y, that there may not be enough Y cells to nodulate the hosts for which it is specific (Roughley, 1970).

## 2.4 The Variable Qualities of Rhizobia

Strain selection is primarily made on the effectiveness, i.e., dinitrogen fixation, of the symbiosis under greenhouse conditions. Determining if a strain of Rhizobia displays a wide-spectrum of specificity is also a significant factor in strain selection. There are several other

characteristics which should also be considered when making a selection. A strain's survival as inocula and on the seed after inoculation must be considered. Ecological factors to be considered which affect both survival and nodulation are soil pH, soil moisture content, root temperature, soil type, pesticide tolerance, and antagonistic organisms. Two other important considerations are the strains' invasiveness and persistance in the soil such that clover reestablishment is successful. Brockwell, et al., (1968) maintained that "it would appear that the chances for finding a single strain of R. trifolii manifesting all the desirable characteristics in association with all agronomically useful species of Trifolium are most remote. It is predicted that there will be an interesting trend toward specialized inoculants for particular species, subspecies, or cultivars using different strains of rhizobia for each." Thus, an inoculum product which contains one or more broad spectrum strain of rhizobia and is claimed to be able to nodulate effectively several cultivars of various clover species may not be equally effective on all clover species.

## 2.5 Commercial Production of an Effective Rhizobia Strain

To be of commercial value, a proven effective strain of rhizobia must be able to be grown in large quantities (Roughly, 1976). Since many of the fast growing rhizobia

such as R. trifolii tend to mutate (Vincent, 1944) a continuous culture system ought not to be used. Instead each fermentation ought to be based on a single batch system (Roughley, 1970).

## 2.6 Methods of Inoculation

There are three major methods of inoculation. First is direct inoculation of the seed with a suspension of a pure culture of rhizobia. This method has been replaced by mixing a pure culture of rhizobia with a peat-based carrier (Burton, 1967). Today "peats and soils high in organic matter are most commonly used as carriers" (Roughley, 1976). In addition to this more generally used second method, a third has developed which has been demonstrated to facilitate clover establishment. This is the use of preinoculated seeds which have been lime pelleted.

The primary reason for abandoning direct application of a pure culture of rhizobia on seeds was the fact that survival was very low (Burton, 1965). In fact, it was shown that the seed coats of some legumes such as subclover contain water soluble antibiotics to which many rhizobia are sensitive (Thompson, 1960; Bowen, 1961).

Dusting seeds with a peat-based inoculum has proven to be unsatisfactory (Roughley, 1970). Date (1970) recommends that the peat inoculum be applied as a slurry. Sus-

pending the peat inoculum in a 10% sucrose solution enhances survival (Vincent, 1958). Pelleting the seed with calcium carbonate has also increased the survival of rhizobia under adverse conditions (Loneragan, 1955). An adhesive material is used when seed is pelleted. Gum arabic 45% w.v. in water was demonstrated to be a better promoter of survival than other adhesives such as methylethyl cellulose (Brockwell, 1962), carbopol, carboxymethyl cellulose, and hydroxypropyl cellulose (Date, 1968). Optimal physical quality of pelleted seed was obtained by using a 40% solution of gum arabic and calcium carbonate which had passed through a 300 mesh sieve (Roughley, et al., 1966).

## 2.7 Survival and Cell Numbers as Factors in Effective Nodulation

The problem of survival of rhizobia after inoculation is directly related to the number of viable rhizobia which are present around or on the seedling during the time in which the plant develops infection sites. It is important for legume establishment that nodules form as soon as possible and dinitrogen fixation is initiated since the seedling may become significantly weakened under conditions of low available nitrogen (Date, 1970). If competition between inoculum and naturalized strains is keen for the plant's initial infection

sites it seems reasonable that a high number of a particular rhizobia in the inoculum would increase the probility of infection by that strain. According to Burton (1967), in the U.S. there is a standard minimum of $10^9$ cells per ml in the broth culture. In Australia, $5 \times 10^8$ cells per ml is minimal for broth cultures intended for non-sterilized peat, and $10^6$ cells per ml is minimal for broth cultures intended for sterile peat (Roughly, 1968). Depending on conditions in the soil environment, the number of cells per seed for successful nodulation ranges from 100 to 100,000. In Australia, the standard minimum number of cells per seed at time of sowing is 300 (Date, 1970). For subclover Ireland and Vincent (1968) found that the number of cells per seed necessary for nodulation by the inoculum strain be equivalent to the number of invasive rhizobial strains per gram of soil.

## 2.8 Survival and Peat-Based Inoculants

Many investigators (Burton, 1965; Vincent, et al., 1962) have demonstrated that peat-based inoculants are superior to liquid cultures. Strijdom and Deschodt (1976) maintain that "liquid cultures seem to lack the protective effect afforded by peat to the rhizobia on seed following inoculation." Protection from dehydration and direct contact with toxic substances on the seed coat are the

primary factors involved (Vincent, et al., 1962).

Many carrier materials have been investigated. Jensen (1961) demonstrated that soil enriched with nutrients supported the survival of rhizobia. Other materials such as soil plus wood-charcoal (Gunning and Jordon, 1954) and a corn-cob-soil-nutrient mixture (Corbey, 1976) have been tested. After assessing the literature, Strijdom and Deschodt (1976) concluded that peat is superior to other carriers and ought to be used if possible. Alternatives should be used only when absolutely necessary.

The variable nature of the peat may affect the survival of the rhizobia, and many of these variables involved in survival of rhizobia have been investigated (Roughley and Vincent, 1967; Roughley, 1968): (i) Source of the peat--certain kinds of peat may be toxic to rhizobia or otherwise fail to promote the survival of rhizobia. (ii) pH--peats are usually acidic and need to be neutralized; this is done with an appropriate amount of calcium carbonate. (iii) Drying and milling of the peat--to prevent the formation of toxic compounds, drying temperatures should not exceed 100$^{o}$C, although Burton (1967) maintained that flash drying the peat carrier with 650$^{o}$C air produced a high quality peat carrier. Generally best results are obtained from peat which passes through a 200 mesh sieve; however, Van Schreven (1970) had satisfactory results

with peat carriers passing through a 2 mm sieve. (iv) Moisture content--because of the variation in organic matter content of various peats suitable as carriers, there is a range of 40-55% final moisture content which affords best survival rates. Burton (1967) reported that a final moisture content of 34-40% resulted in good survival. (v) Sterilization of peat--this favors viability during storage. In the U.S. sterilization is never entirely complete. Flash drying almost completely sterilizes the peat but "no difficulties have been experienced with flash-dried peat used in the U.S." (Burton, 1967). On the whole, complete sterilization is recommended for the benefit of initial multiplication of the rhizobia and their survival during storage. Strijdom and Deschodt (1976) and Roughley and Vincent (1967) found that sterile peat was superior to non-sterile peat. Gamma radiation has proven to be the most effective method of sterilization. The high temperature of autoclaving sometimes leads to the formation of toxins in the peat.

## 2.9 Containers and Storage of Peat Carrier Inocula

The need for gas exchange and moisture retention during storage (Van Schreven, et al., 1954; Roughley, 1968) entails the use of appropriate containers. Though bottles plugged with cotton and wrapped in cellophane have been used

successfully (Van Schreven, 1958), polythene bags have proven to be successful containers of peat carrier inoculum for commercial distribution (Roughley, 1970).

The effects of temperature and moisture loss are more acute for non-sterile peat inocula (Roughley, 1970). For sterile peat inocula, Roughley (1968) showed that maximum numbers were obtained after 4 months if the peat inoculum was initially incubated at 26°C for one week and then at 4°C for 3 weeks. Pure cultures in sterilized peat may be stored for 6 months at 4°C followed by 9 months at retail conditions; for non-sterile peat, these storage periods are halved (Roughley, 1970). Preinoculated, pelleted seed should be sown right after pelleting; maximum storage at 15°C is 2-4 weeks; the viability of rhizobia becomes more variable after 10 days of storage at 15°C (Roughley, 1970; Date, 1970).

## 2.10 Testing Commercial Inocula

How are these commercial inocula to be tested? Leonard (1944) maintained that "under practical conditions commercial inoculants are used in the field and it would seem advisable to test them under similar conditions. Field testing is unsatisfactory on account of several factors, among which may be mentioned the almost universal presence of some species of nodule bacteria and varying climatic and

soil conditions, some of which cannot be controlled." Thus Leonard recommended the abandonment of field trials for the controlled confines of the green-house. It cannot be overlooked, however, that though an inoculum may perform well in green-house conditions, it would be considered worthless by farmers if the inoculum failed in the field.

The factors which Leonard dismisses are those which most affect the survival, establishment and persistence of rhizobial inoculants. Thus Vincent (1970) maintains that although "green house experiments are valuable for the primary assessments of the symbiotic capacity of particular rhizobium/host combinations the full evaluation depends on the field trials." Leonard (1944) and Vincent (1970) both agree that, for the green-house and field trials, the main points to observe in determining the effectiveness of inocula are the differences between treated and control plants in dry matter, color, size, nodule formation, and percent nitrogen.

Additional factors may also be investigated and quantified which would reflect on the suitability and success of commercial inocula. For example, for peat-based inocula the number of viable cells per gram of peat, pH, percent moisture content, and sterility of the peat would reflect the potential effectiveness of the inoculum. Knowing the number of viable cells per seed after inocula-

tion would give one a close estimate regarding the survival potential of the rhizobia in the soil and nodulation of the host, especially when the number of competitors is significant. For multi-host inocula, enumerating rhizobia specific for the particular cultivars may be necessary in order to obtain a total count of rhizobial cells. Is there the possibility of ineffective strains out-competing effective strains in the same multistrain inoculant and thus reflecting possible lack of establishment? Are the inoculants wide-spectrum enough to include the cultivar to be inoculated? Such questions are addressed in this work, and investigated through both green-house and field trails.

# BIBLIOGRAPHY

Allen, O. N. 1973. Symbiosis: rhizobia and leguminous plants. p. 98-104. In: Heath, M. E., Metcalfe, D. S., and Barnes, R. F., (ed.). Forages the science of grassland agriculture, Iowa State University Press, Ames.

Bowen, G. D. 1961. The toxicity of legume seed diffusates toward rhizobia and other bacteria. Plant and Soil. 15:155-165.

Brockwell, J. 1962. Studies on seed pelleting as an aid to legume seed inoculation. Austr. J. Agric. Res. 13:638-649.

Brockwell, J., Dudman, W. F., Gibson, A. H., Hely, F. W., and Robinson, A. C. 1968. An integrated program for the improvement of legume inoculant strains. Ninth International Congress of Soil Science Transactions, 2:103-114.

Burton, J. C. 1965. The rhizobium-legume association. p. 107-134. In: Gilmour, G. M., and Allen, O. N. (ed.). Microbiology and soil fertility. Oregon State University Press, Corvallis.

Burton, J. C. 1967. Rhizobium culture and use. p. 1-30. In Peppler, H. J. (ed.). Microbiol Technology. Rheinhold Publishing Co., New York.

Burton, J. C., and Allen, O. N. 1949. Inocualtion of crimson clover (Trifolium incarnatum L.) with mixtures of rhizobia strains. Soil Sci. Soc. Amer. Proc. 14:191-195.

Cameron, D. G. 1959a. Subterranean clover and soil conservation. J. Soil Conserv. M. S. Wales. 12:5-18.

Cameron, D. G. 1959b. Subterranean clover and soil conservation. II. J. Soil Conserv. N. S. Wales. 12:105-124.

Corbey, N. D. L. 1976. A method of making a pure culture, peat-type, legume inoculant, using a substitute for peat. p. 169-173. In: Nutman, P. S. (ed.) Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge.

Date, R. A. 1970. Microbiological problems in the inoculation and nodulation of legumes. Plant and Soil. 32: 703-725.

Date, R. A. 1976. Principles of Rhizobium strain selection. p. 137-150. In: Nutman, P. S. (ed.). Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge.

Gunning, C. and Jordan, D. C. 1954. Studies on humus type inoculants. II. Preparational effectivity. Canadian J. of Agric. Sci. 34:225-233.

Hagedorn, C. 1978a. Crop production and soil fertility as influenced by nitrogen-fixation in legumes. Proceedings, 29th Annual Fertilizer Conference of the Pacific Northwest, Beaverton, Oregon; July 11-13, 1978.

Hagedorn, C. 1978b. Nodulation of Trifolium subterraneum L. by introduced Rhizobia in southwest Oregon soils. Soil Sci. Soc. Amer. J. 43:515-519.

Ireland, J. A., and Vincent, J. M. 1968. A quantitative study of competition for nodule formation. Ninth International Congress of Soil Science Transactions, 2:85-93.

Jensen, H. L. 1961. Survival of Rhizobia meliloti in soil culture. Nature. 192:682-683.

Leonard, L. T. 1944. Method of testing legume bacteria cultures and results of tests of commercial inoculants in 1943. U.S.D.A. Circular No. 703.

Loneragen, J. F., Meyer, D., Fawcett, R. G., Anderson, A. J. 1955. Lime pelleted clover seeds for nodualtion on acid soils. Journal Austr. Institute Agric. Sci. Dec. 1955, p. 264-265.

Means, U. M. Johnson, H. W., and Erdman, L. W. 1961. Competition between bacterial strains effecting nodualtion in soybeans. Soil Sci. Soc. Amer. Proc. 25:105-108.

Moreley, F. H. W. 1961. Subterranean clover. Advances in Agron. 13:57-123.

Rampton, H. H. 1945. Growing subclover in Oregon. Oregon Agriculture Experiment Station Bull. 432.

Roughley, R. J. 1968. Some factors influencing the growth and survival of root-nodule bacteria in peat cultures. J. Appl. Bact. 31:259-265.

Roughley, R. J. 1970. The preparation and use of legume seed inoculants. Plant and Soil. 32:675-701.

Roughley, R. J. 1976. The production of high quality inoculants and their contribution to legume yield. p. 125-136. In: Nutman, P. S. (ed.). Symbiotic Nitrogen Fixation in Plants. Cambridge University Press, Cambridge.

Roughley, R. J., Date, R. A., and Walker, M. H. 1966. Inoculating and lime-pelleting legume seed. Agric. Gazette of N. S. Wales. 77:143-146.

Roughley, R. J., Vincent, J. M. 1967. Growth and survival of Rhizobium spp. in peat culture. J. Appl. Bact. 30:362-76.

Simpson, J. R. 1965. The transference of nitrogen from pasture legumes to an associated grass under several systems of management in pot culture. Austr. J. Agric. Res. 16:915-26.

Steinborn, J. and Roughley, R. J. 1974. Sodium chloride as a cause of low numbers of Rhizobium in legume inoculants. J. Appl. Bact. 37:93-99.

Strijdom, B. W., Deschodt, C. C. 1976. Carriers of rhizobia and the effects of prior treatment on the survival of rhizobia. p. 151-153. In: Nutman, P. S. (ed.). Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge.

Thompson, J. A. 1960. Inhibition of nodule bacteria by an antibiotic from legume seed coats. Nature. 167: 619-620.

Van Schreven, D. A. 1958. Methods used in the Netherlands for the production of legume inoculants. p. 328-333. In: Hallsworth, E. G. (ed.). Nutriton of Legumes. Butterworth and Co. London.

Van Schreven, D. A. 1970. Some factors affecting growth and survival of Rhizobium spp. in soil-peat cultures. Plant and Soil. 32:113-130.

Van Schreven, D. A., Otzen, D., Lindenbergh, D. J. 1954. On the production of legume inoculants in a mixture of peat and soil. Antonie van Leeuwenhoek, Journal of Microbiology and Serology. 20:33-57.

Vincent, J. M. 1944. Variation in the nitrogen fixing property of Rhizobia trifolii. Nature. 153:496-497.

Vincent, J. M. 1958. Survival of the root-nodule bacteria p. 108-123. In: Hallsworth, E. G. (ed.) Nutrition of the legumes. Academic Press Inc., New York.

Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publications, Oxford.

Vincent, J. M., Thompson, J. A., and Donovan, K. O. 1962. Death of root-nodule bacteria on drying. Austr. J. Agric. Res. 13:258-270.

# THE EFFECTS OF COMMERCIAL *RHIZOBIUM* INOCULANTS ON THE ESTABLISHMENT OF *TRIFOLIUM SUBTERRANEAN* IN SOUTHWEST OREGON SOILS

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# 3. INTRODUCTION

Since Rampton (1945) discussed the agronomic significance of Trifolium subterraneum (or subterranean clover, or subclover) and its suitability for Oregon, west of the Cascades, interest in subclover has increased in southwest Oregon (Hagedorn, 1978b). A major problem in successfully establishing subclover has been with the quality of rhizobial inoculants available for application (Hagedorn, 1978a). A question to be investigated in this paper is how effective are the available commercial rhizobial inoculants?

Significant differences between products may affect plant response to inoculation. A multiple-host inoculant may be comprised of one or several strains of Rhizobium trifolii. Investigators (Burton, 1965; Burton and Allen, 1949; Brockwell, et al., 1968; Allen, 1973) have demonstrated that strains of R. trifolii tend to be fairly specific, and that their potential for a wide-spectrum of specificity probably is minimal. Roughley (1970) has shown that competition between strains in multistrain inocula increases the unpredictability of inoculum quality. If a multihost inoculant is to benefit the rancher, it must not include a rhizobial strain which ineffectively nodulates the cultivar for which the inoculum is prepared

(Burton, 1967). Problems such as ineffective nodulation are probable when multihost and multistrain rhizobial inoculants are applied to subclover seeds.

Lime pelleting inoculated subclover seed increases the survival rate of the inoculum strain (Lonevagen,1955; Roughley, et al., 1968; Roughley, 1970; Date, 1970). Ideally, the inoculum used for pelleted seed is comprised of a pure strain which has demonstrated high effectiveness for the particular cultivar. Thus, preinoculated lime pelleted Mt. Barker seed, may be less likely to fail either in field trials or laboratory tests.

The most significant factor, other than that of range of specificity, affecting the success of both peat-based inocula and preinoculated, lime-pelleted seed is the numbers of viable R. trifolii cells on the seed at time of sowing (Burton, 1967; Roughley, 1968; Date, 1970). For practical application, Ireland and Vincent (1968) maintain that the number of cells per seed necessary for nodulation by the inoculum strain be equivalent to the number of invasive rhizobial strains per gram of soil. Other parameters affecting the success of a peat-based inoculum are the pH, percent moisture and sterility of the peat carrer (Roughley and Vincent, 1967; Roughley, 1968).

The object of this paper will be 1) to determine the pH, percent moisture and extent of contamination of the peat-based inocula; 2) to determine the number of viable rhizobial cells per unit of inoculum and per seed after inoculation; 3) to determine if the inocula are single or multistrain; 4) determine the effectiveness of the available commercial products under field conditions.

# 4. MATERIALS AND METHODS

## 4.1 Seed and Commercial Products Tested

The commercial inoculants evaluated in this study were obtained from county extension agents who purchased the products at local seed dealers. The products were refrigerated at $4^{o}C$ as soon as they were received, and all laboratory analyses were initiated soon afterwards. The three products evaluated are Nitragin (Milwaukee), a peat-based inoculum prepared for rose and subterranean clovers, Northrup-King (Minneapolis), another peat-based inoculum prepared for alfalfa and clovers, and Celpril (Mantica, CA) a pre-inoculated, lime pelleted Mt. Barker subclover seed.

Inoculation of seed with the peat-based products was done according to directions on their respective polyethylene packages. The directions for Nitragin called for an initial slurry by mixing the peat inoculum in 946 ml. of water (distilled water was used). The recommended application rate used was $2.09 \times 10^{-2}$ ml of slurry/g seed. Northrup-King called for applying directly (i.e., dusting) the contents in the package on one bushel of seed, or $3.78 \times 10^{-3}$g inoculum/cc seeds. The Mt. Barker subclover seeds were placed in polyethylene whirl-pack bags; the inoculum preparation was measured, aseptically applied

and mixed thoroughly the night before planting. The inoculated seed was stored at 4°C for approximately 12 hours until transit to the experimental sites.

Certified Mt. Barker subclover seeds were used. The percent germination for the Mt. Barker subclover seed and the pelleted product was determined by sprouting 100 seeds on a plate of sterile water agar at two different temperatures, 10°C and 25°C. The average of four plates was taken for each sample of seed. The final reading was taken after 12 days.

## 4.2 Site Description and Design

Three of the four sites used in this study are located in Coos County, the fourth is in Douglas County. The McKenzie site is situated at the extreme upper slope of a spur ridge in the mountainous uplands immediately east of the coast-line. The plots were on a 3-5% slope. The Geaney site is situated midslope on a hill side facing west. The plots were on a 15% slope. The Carmen site was located on the eastern slopes draining into the south fork of the Coquille River. The hillside was midslope. Plots were on a 1-2% slope. The Metz Hill site in Douglas County is situated in rolling hills. The site itself is on flat land which has been sown to wheat. Soil analyses were made of samples from each site (Table 1). The

analyses were conducted by the soils testing laboratory at Oregon State University (Berg and Gardner, 1978).

All plots were 1.83 m by 1.23 m except for plots at site 1, which had dimensions of 1.52 m by 1.23 m. There were duplicate plots per treatment. Each plot was separated by a 0.91 m buffer zone. A randomized plot design was not used since control of possible interplot contamination took precedence (Vincent, 1970). Thus at each site the treatments were arranged in two columns with rows of duplicate treatments, one row per treatment.

The plots were staked out as described above on a prepared seed bed. Each plot was amended with 44.7 kg P/ha., 22.3 kg S/ha., 111.5 kg K/ha and 1.7 kg Mo/ha. For the uninoculated plus-nitrogen controls plots, 112 kg/ha nitrogen was added. The number of naturalized Rhizobia trifolii in the soil at time of planting for each site was determined by the 2 tube/dilution plant infection procedure, using Mt. Barker subclover as host (Vincent 1970).

All treatments except for Celpril and the controls, were sown at a seeding rate of 136 kg seed/ha. Celpril pelleted seed was sown at a rate of 196, 98, 49, and 25 kg seed/ha. the uninoculated controls were seeded at 68 kg seed/ha. The difference between Celpril and the other unpelleted Mt. Barker seed is based on the fact that the equivalent number of seeds per gram of seed

are different, i.e., 160 seeds/gr seed for unpelleted Mt. Barker to 110 seeds/gr. seed of Celpril pelleted seed. The seed was sown in eight rows per plot at all sites except site 1 which was sown in seven rows per plot. All care was taken in order to prevent intertreatment contamination.

The sites were planted in the first week of October. Because of the unusually adverse weather conditions, a drought in the fall and the coldest winter recorded in Oregon, the first sampling occurred during the last week in February. The second, and final samplings occurred in the first week of April and the second week in May. Ten samples of two plants per sample were randomly taken from each plot. The tops of the sample plants were dried at $60^{o}C$ for 72 hours and the dried plant tissue was then weighed on an analytical balance. Percent total plant nitrogen was determined for the final sampling only by the macro-kjeldahl method (Berg, and Gardner, 1978). The sample analyzed was a blend of the 20 plants sampled per plot.

## 4.3 Laboratory Analysis

Percent moisture of the peat-based products was determined by putting a 10g sample of each product in a 50 ml beaker previously oven dried. The beaker containing the

peat inoculum was weighed and then oven dried at 60°C for 48 hours, and the percent moisture then determined. pH determination of peat inocula was made using methods described by Roughley and Vincent (1967).

Counts of the viable R. trifolii effective for Mt. Barker subclover in the peat-based inocula, and the subclover seed after inoculation with the appropriate quantity of Nitragin and Northrup-King products and Celpril product were determined by the 2 tube/dilution plant infection procedure as described by Vincent (1970). The pelleted seeds and the inoculated seed were blended in diluent in a Waring blender at medium speed for 3 minutes before being further diluted for the plant infection test. Counts of viable cells of R. trifolii which effectively nodulate species of clover other than subclover were determined following the same 2 tube/dilution plant infection procedure; rose clover was used for Nitragin and red and New Zealand white clovers were used for Northrup-King. Dilutions of each peat-based product (using a sterile 0.05% peptone dilutent) were plated on Congo Red-YMA which conformed with Vincent's (1970) recommendations. Thus counts of rhizobial and non-rhizobial cells were obtained.

Isolation of strains was obtained by excising nodules from the subterranean, rose, red, and New Zealand white

clover seedlings all of which were grown as part of the plant infection procedure. The excised nodules were sterilized as recommended by Vincent (1970), squashed and streaked on YMA plates. Further streaking on YMA plates was done until pure colonies were obtained. Isolates were stored on YMA screw-cap slants at $4^{o}C$. Four subclover seedlings per isolate were inoculated with 1 ml of broth containing at least $10^{9}$ cells/ml. Two controls of rose, red, and New Zealand white clovers were used for isolates obtained from those species of clover. Four seedlings were used for both the (-)N and +N uninoculated controls. One ml. of sterile 0.05% $KNO_3$ was applied every seven days to the +N uninoculated controls.

# 5. RESULTS AND DISCUSSION

The application of P, K, S, and Mo, at rates commensurate with those recommended in the Oregon State Extension Service fertilizer guide for subclover-grass pasture (1973) decreases the probability that the availability of nutrients affected responses to treatments. All sites are acidic; and percent base saturation is generally low. The McKenzie site has the lowest pH and percent saturation; these factors may be related to the overall lower yields obtained for all treatments at that site (Table 2). Since the soil, weather and geographical difference between sites are many and complex, further site comparisons will not be made. The important point to notice is whether or not the same treatment performed consistently better than the others.

Samples for the lower seeding rates for Celpril were not taken at any site on the first two sampling periods so that there would be enough plants for the last sampling period. There is no data for the lowest seeding rate for Celpril because by the last sampling period there were less than 20 plants per plot. Data for the Carmen site are not presented (Tables 2 and 3) because the data for the first two sampling periods were insufficient and by the last sampling period the growth of volunteer subclover obscured all the treatments.

No significant differences in dry weights are seen between (-)N and +N uninoculated controls, though the +N controls show consistently higher yields (Table 2). Both (-)N and +N controls were nodulated at all sites. Though the populations of R. trifolii effective for Mt. Barker subclover at each site are considered low for effective nodulation (Vincent, 1970) the lack of significant differences in dry weights between controls seems to demonstrate a moderate effectiveness of the naturalized rhizobia.

There are differences in dry weights between Nitragin and Northrup-King at single strength and Celpril at the highest seeding rate (Table 2). At the McKenzie site the only significant difference in dry weights is seen at the first sampling period with Northrup-King showing the greatest yield; but by the last sampling period although there were no significant differences between treatments, Northrup-King showed the lowest yield and the Celpril pelleted seed showed higher yields. At the Geaney site, the Celpril product showed significantly higher dry weights than the Northrup-King product for all sampling periods, and than the Nitragin product for the first and last sampling periods; Nitragin showed significantly higher dry weights than the Northrup-King product for the last two sampling periods. At the Metz Hill site

treatment differences were seen only for the last sampling period; there were no differences between Nitragin and Celpril, but both were significantly higher than Northrup-King.

There were no significant difference between recommended and double the recommended rates for the peat-based products. The number of viable rhizobia per seed (Table 5) are low, and negligible for the Northrup-King product. Thus, doubling the recommended amount of inoculum per unit of seed essentially did not effect an increase in dinitrogen fixation.

The dry weight data (Table 2) for Celpril indicates that both seeding rate and treatments had an effect on responses. Only at the Geaney site was there significant difference between the (-)N uninoculated control and the Celpril product at half the highest or comparable seeding rate. There were no significant differences between the seeding rates except at the Geaney site at the third sampling. The general trend was an increase in dry weight with a decrease in seeding rate. The plants at the lower seeding rates seemed to have been more adversely affected by the inclement winter weather (the coldest December and January recorded in Oregon). Poor germination probably was not a factor (Table 6). Though the plants at the lower seeding rates tended to be larger, it appeared from visual

observation that the yield per unit area decreased with decreased seeding rates. The lowest seeding rate for Celpril, 25 kg seed/ha. appears to be inadequate for establishment of subclover.

The data on percent plant nitrogen (Table 3) show insignificant treatment differences at the Metz Hill site, but significant differences for the other sites. At the McKenzie site, Celpril, at all seeding rates, shows a significantly higher nitrogen content than the (-)N uninoculated control and the peat-based treatments. At the Geaney site, only the Celpril pelleted product at the highest seeding rate shows a significantly higher nitrogen content than the Northrup-King treatments. Though the dry weight differences at the McKenzie site were insignificant, the percent total plant nitrogen data indicate that the Celpril product was effectively fixing dinitrogen.

The field data indicated that the Celpril preinoculated, pelleted, Mt. Barker subclover seed out-performed both peat-based products with the exception of Nitragin which out-performed the other products at the Metz Hill site. It is also apparent that Nitragin was usually more effective than the Northrup-King peat-based product.

Laboratory analyses indicate that low numbers of rhizobia effective on Mt. Barker subclover in the peat (Table 7), contamination of the peat (Table 8), and a low

cell count per seed (Table 5) may have attributed greatly to the poor response of the peat-based products. The pH of the peat of both products was neutral and therefore satisfactory; but percent moisture was below the optimal range of 40-60 percent for both. Competition with contaminants and other rhizobial strains may have attributed to the low counts of rhizobia effective for Mt. Barker subclover. The population of rhizobial strains effective for rose clover in the case of the Nitragin product, and other clovers such as red and New Zealand white, and vernal alfalfa in the case of the Northrup-King product (Table 9) were low.

If the Nitragin product contains a wide-spectrum strain of R. trifolii, isolates from nodules excised from either subclover or rose clover should effectively nodulate seedlings of both cultivars. Only one out of three isolates originating from the Nitragin product, isolate 5, displayed significant effectiveness (Table 10). All other isolates either showed little or no effectiveness. The only surviving isolate from the Celpril pelleted product seems to have lost its effectiveness. Isolation and storage conditions may have adversely affected the effectiveness of all the isolates, but this is unlikely (Vincent, 1954).

One of the isolates from rose clover effectively nodulated subclover (Table 11), but the other two isolates

ineffectively nodulated Mt. Barker subclover. None of the isolates from red or New Zealand white clovers effectively nodulated Mt. Barker subclover. Thus it seems evident that both peat-based inoculants contain strains which ineffectively nodulate Mt. Barker subclover. This fact demonstrates the problems with multihost and multistrain inoculants, and is probably related to poor field performance.

Both laboratory analyses and field trials have illustrated problems which arise with multihost, multistrain peat-based inocula; the probability of ineffective nodulation occuring is increased by using multistrain inoculants. The reliability of a pelleted product may indicate that ineffective nodulation is less likely to occur when preinoculated, lime-pelleted seed is used. The main problem with preinoculated pelleted clover seed is its short shelf life. It must be sown as soon as possible after inoculation.

# LITERATURE CITED

Allen, O. N. 1973. Symbiosis: rhizobia and leguminous plants. p. 98-104. In Heath, M. E., Metcalfe, D. S., and Barnes, R. F. (ed.) Forages the science of grassland agriculture, Iowa State University Press, Ames.

Berg, M. G., and Gardner, E. H. 1978. Methods of soil analysis used in the soil testing laboratory at Oregon State University. Oregon State Agricultural Experiment Station, Special Report 321.

Burton, J. C. 1965. The rhizobium-legume association. p. 107-134. In Gilmour, G. M., and Allen, O. N. (ed.) Microbiology and soil fertility. Oregon State University Press, Corvallis.

Burton, J. C. 1967. Rhizobium culture and use. p. 1-33. In Peppler, H. J. (ed.) Microbial Technology. Rinehold Publishing Co., New York.

Burton, J. C., and Allen, O. N. 1949. Inoculation of crimson clover (Trifolium incarnatum L.) with mixtures of rhizobia strains. Soil Sci. Soc. Amer. Proc. 14:191-195.

Brockwell, J. 1963. Accuracy of a plant infection technique for counting population of Rhizobium trifolii. Appl. Microbiol, 11:377-383.

Brockwell, J., Dudman, W. F., Gibson, A. H., Hely, F. W. Robinson, A. C. 1968. An integrated program for the improvement of legume inoculant strains. Ninth International Congress of Soil Science Transactions, 2:103-114.

Date, R. A. 1970. Microbiological problems in the inoculation and nodulation of legumes. Plant and Soil, 32:703-725.

Hagedorn, C. 1978a. Crop production and soil fertility as influenced by nitrogen-fixation in legumes. Proceedings, 29th Annual Fertilizer Conference of the Pacific Northwest, Beaverton, Oregon; July 11-13, 1978.

Hagedorn, C. 1978b. Nodulation of Trifolium subterraneum L. by introduced rhizobia in south west Oregon soils. Soil Sci. Soc. Amer. J. 43:515-519.

Ireland, J. A., and Vincent, J. M. 1968. A quantitative study of competition for nodule formation. Ninth International Congress of Soil Science Transactions, 2:85-93.

Loneragen, J. F., Meyer, D., Fawcett, R. G., Anderson, A. J. 1955. Lime pelleted clover seeds for nodulation on acid soils. J. Austr. Inst. Agric. Sci., Dec., 1955, p. 264-265.

Oregon State University Extension Service. 1973. Fertilizer guide for subclover-grass pastures. Oregon Extension Service, Circ. FG 4, 2 p.

Rampton, H. H. 1945. Growing subclover in Oregon. Oregon Agric. Experiment Station Bull. 432.

Roughley, R. J. 1968. Some factors influencing the growth and survival of root-nodule bacteria in peat culture. J. of App. Bact., 31:259-265.

Roughley, R. J. 1970. The preparation and use of legume seed inoculants. Plant and Soil, 32:675-701.

Roughley, R. J., and Vincent, J. M. 1967. Growth and survival of Rhizobium spp. in peat culture. J. App. Bact., 30:362-376.

Vincent, J. M. 1954. The root-nodule bacteria of pasture legumes. Proc. Linn. Soc. N. S. Whales, 79:iv-xxxii.

Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publications, Oxford.

TABLE 1. Soil Characteristics of Trial Sites at Time of Sowing.*

| Field Site | Soil Classification | ppm P | ppm K | meq/100g Ca | meq/100g Mg | meq/100g Na | % Total N | µg/g $NO_4^+$-N $NO_3^-$-N | CEC | % Base Saturation | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| McKenzie | clayey, mixed mesic Typic Dystrochrepts | 24 | 208 | 4.05 | 0.99 | 0.39 | 0.49 | 9.5 | 27.62 | 26.90 | 5.1 |
| Geaney | fine, mixed, mesic Typic Dystrochrepts | 9 | 368 | 8.8 | 6.9 | 0.28 | 0.25 | 13.6 | 34.16 | 49.5 | 5.5 |
| Carmen | fine, mixed, mesic Typic Dystrochrepts | 53 | 604 | 13.4 | 4.4 | 0.17 | 0.28 | 12.3 | 25.18 | 77.92 | 5/6 |
| Metz Hill | fine, mixed, mesic Ultic Haploxeralf | 19 | 298 | 6.2 | 2.6 | 0.64 | 0.26 | 14.6 | 19.4 | 52.58 | 5.3 |

* All analyses are from the top 15 cm of soil at each site.

TABLE 2. Subclover Mean Dry Weights (mg) on Inoculated Plots and (-)N and +N Uninoculated Controls.

| Site | Sampling period | Northrup-King recommended rate | Northrup-King twice recommended rate | Northrup-King recommended rate | Northrup-King twice recommended rate | Celpril 196 kg seed/ha | Celpril 98 kg seed/ha | Celpril 49 kg seed/ha | Celpril 25 kg seed/ha | Uninoculated Controls (-)N | Uninoculated Controls +N | L.S.D .05 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| McKenzie | 1 | 44 | 42 | 94 | 42 | 75 | N.D. | N.D. | N.D. | 85 | 74 | 21 |
| | 2 | 243 | 191 | 223 | 178 | 377 | N.D. | N.D. | N.D. | 175 | 289 | N.S. |
| | 3 | 1265 | 927 | 669 | 702 | 1477 | 1911 | 1993 | N.D. | 947 | 1046 | N.S. |
| Geaney | 1 | 41 | 26 | 30 | 33 | 60 | N.D. | N.D. | N.D. | 35 | 70 | 20 |
| | 2 | 323 | 182 | 148 | 155 | 381 | N.D. | N.D. | N.D. | 176 | 238 | 91 |
| | 3 | 845 | 658 | 471 | 366 | 1349 | 1695 | 2536 | N.D. | 1096 | 1346 | 368 |
| Carmen | No significant data | | | | | | | | | | | |
| Metz Hill | 1 | 48 | 62 | 48 | 22 | 59 | N.D. | N.D. | N.D. | 23 | 43 | N.S. |
| | 2 | 405 | 542 | 497 | N.D. | 585 | N.D. | N.D. | N.D. | 246 | 253 | N.S. |
| | 3 | 4812 | 5794 | 2420 | 1296 | 4702 | 4980 | 5444 | N.D. | 3166 | 3758 | 2016 |

TABLE 3. Percent Total N in Subclover Plants on Inoculated Plots and Uninoculated Controls for the Last Sampling Period.

| Site | Northrup-King recommended rate | Northrup-King twice recommended rate | Northrup-King recommended rate | Northrup-King twice recommended rate | Celpril 196 kg seed/ha | Celpril 98 kg seed/ha | Celpril 49 kg seed/ha | Celpril 25 kg seed/ha | (-)N | +N | L.S.D. .05 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| McKenzie | 2.31 | 2.39 | 2.39 | 2.50 | 2.98 | 2.81 | 2.94 | N.D. | 2.27 | 2.60 | 0.36 |
| Geaney | 2.43 | 2.46 | 2.02 | 2.03 | 2.80 | 2.67 | 2.57 | N.D. | 2.37 | 2.49 | 0.51 |
| Carmen | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Metz Hill | 2.14 | 1.99 | 1.81 | 1.26 | 2.35 | 2.12 | 2.06 | N.D. | 1.94 | 2.00 | N.S. |

TABLE 4. Number of Naturalized Rhizobia Effective for Mt. Barker Subclover at Each Sampling Site.

| Site | Number of Viable Cells gram soil$^{-1}$ |
|---|---|
| McKenzie | $1.7 \times 10^2$ |
| Geaney | $5.8 \times 10^2$ |
| Carment | $1.7 \times 10^2$ |
| Metz Hill | $1.7 \times 10^0$ |

TABLE 5. Number of Viable Cells Per Seed After Inoculation.

| Product | Number of Cells per seed |
|---|---|
| Nitragin | 18.1 |
| Northrup-King | 1.0 |
| Celpril | 77.3 |

TABLE 6. Percent Germination Rate for Mt. Barker Subclover and Celpril Pelleted Mt. Barker Seed Used in the Field Trials.

| | Temp Incubation | Days Incubation | % Germination |
|---|---|---|---|
| Mount Barker | 25°C | 6 | 89 |
| | 10°C | 14 | 87 |
| Celpril (pelleted Mt. Barker) | 25°C | 12 | 79 |
| | 10°C | 12 | 77 |

TABLE 7. Number of viable Rhizobia trifolii effective for Mt. Barker subclover per gram of peat for the peat inocula.

| Product | Number of Cells gram peat$^{-1}$ |
|---|---|
| Nitragin | $3.7 \times 10^5$ (peat) |
| Northrup-King | $3.0 \times 10^4$ (peat) |

TABLE 8. Count of total Rhizobial cells and other contaminating bacteria in peat inocula as determined by plate counts on Congo Red-YMA medium.

| Product | Total Cells gram peat$^{-1}$ | Total Rhizobial Cells gram peat$^{-1}$ | % Rhizobia |
|---|---|---|---|
| Nitragin | $3.4 \times 10^9$ | $5.4 \times 10^8$ | 15.9 |
| Northrup-King | $5.5 \times 10^9$ | $6.1 \times 10^8$ | 11.1 |

TABLE 9. Number of Rhizobial cells effective for species other than subclover.

| Product | Seed | Number of Cells gram peat$^{-1}$ |
|---|---|---|
| Nitragin | Rose Clover | $8.5 \times 10^5$ |
| Northrup-King | White Clover | $2.9 \times 10^3$ |
| | Red Clover | $8.5 \times 10^3$ |
| | Vernal Alfalfa | $8.5 \times 10^5$ |

TABLE 10. Mean dry weights of subclover seedlings inoculated with pure isolates taken from nodules excised from subclover previously inoculated with a commercial inoculum.

| Origin | Celpril | Northrup-King | | Nitragin | | | | -N Control | +N Control |
|---|---|---|---|---|---|---|---|---|---|
| isolate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Dry Wts. mg | 7.0 | 12.9 | 12.5 | 10.9 | 16.3 | 12.0 | 10.7 | 10.9 | 17.9 |

$L.S.D._{.05}$ = 3.6 mg

TABLE 11. Mean dry weights of subclover seedlings inoculated with pure isolates taken from species of clover other than subclover.

Peat based

| +Product | Nitragin | | | Northrup-King | | | | | | | | Controls | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| host | Rose | Rose | Rose | Red | Red | Red | Red | White | White | White | White | (-)N | +N |
| - isolates | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Dry Wts. (mg) | 17.5 | 12.6 | 8.5 | 12.0 | 7.7 | 11.6 | 9.6 | 10.8 | 8.6 | 7.2 | 13.2 | 11.0 | 18.8 |

$L.S.D._{.05} = 5.4$