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THE EXCISED EMBRYO CULTURE METHOD FOR CONTROLLED SEEDLING GROWTH OF THE SWEET FERN, *COMPTONIA PEREGRINA*, OF THE FAMILY MYRICACEAE[1]

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INTRODUCTION: Sweet fern, *Comptonia peregrina* (L.) Coult. according to Fernald (1950), in older literature often referred to as *Myrica asplenifolia* L. or *Comptonia asplenifolia* (L.) Ait., is the only species in this genus. It is a low branching, stoloniferous shrub up to a meter and a half tall, with linear-lanceolate leaves that are regularly and deeply pinnatifid giving a fern-like appearance, though it is not at all related to the ferns. It has a characteristic sweet resinous scent. The plants are monoecious or dioecious, flowers are in catkins; staminate catkins are slender and cylindrical; pistillate catkins become a spherical bur-like fruit. Because the shrub spreads by rhizomes, it forms clonal colonies. The family, *Myricaceae,* includes but two genera according to Fernald (1950): *Myrica* and *Comptonia.* The closest relative of *Comptonia, Myrica Gale,* is also abundant in this area, especially in swamps north of Lake Superior. Fernald (1950) gave the range of *Comptonia peregrina* as extending from Cape Breton Island, Nova Scotia westward to Manitoba in Canada, and southward and westward in the United States to Virginia, northern Indiana, northeastern Illinois, and Minnesota. Fernald (1950) listed also variety *asplenifolia* being distinguishable mainly by the smaller stature and more minute hairiness or glabrous condition.

DISTRIBUTION IN MINNESOTA: In Minnesota (Figure 1) and elsewhere, sweet fern may occur as a very successful pioneer in dry, acid to neutral, sandy or rocky soil. Its ability to become established and grow rapidly in these infertile mineral soils, suggest that it possesses some mechanism which makes it independent of soil nitrogen. Characteristic nodules similar in appearance to those of alder (*Alnus*) are suspected of fixing nitrogen. These are coralloid masses of modified lateral roots which result from repeated branching. Early in the first season of growth, the nodule is unbranched and cylindrical in

[1] The author wishes to thank Dr. D. B. Lawrence, University of Minnesota for suggesting the problem and for guidance and encouragement in this study.

shape, averaging 3-4 mm. in length, and white to gray in color. Within several months during the growing season, they branch to form three tips and become dark brown in color and woody except at the light colored, apical tips. Review of the literature suggests that any positive classification of the causative organism would be premature. It has been variously reported as belonging to the bacteria, actinomycetes, filamentous fungi, and the slime molds. Artificial culture of the microorganisms has not been successful; this failure suggests that the relationship may be one of an obligate symbiont. Therefore, in order to demonstrate the nitrogen-fixing capability of the nodule symbiont, it is necessary to grow the seedling in sterile culture. The most certain way of providing a supply of seedlings which are free of microorganisms is through sterile culture of excised embryos.

Artificial culture of excised embryos of various plant materials

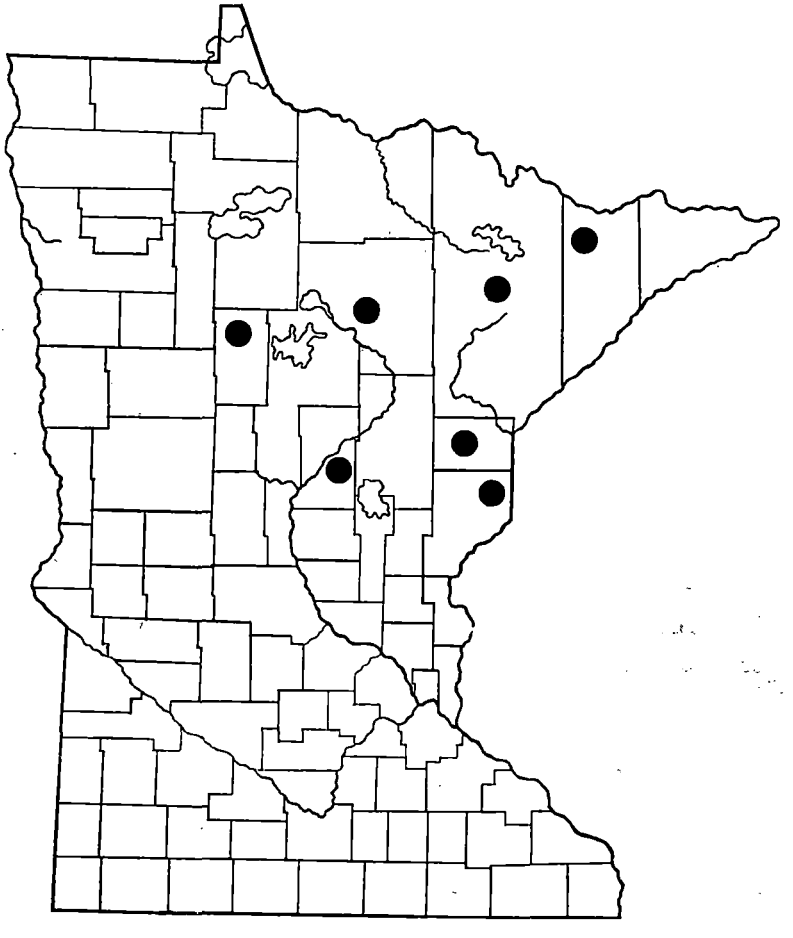

Fig. 1. Distribution of *Comptonia peregrina.*

has been practiced since it was first successfully accomplished by Hannig (1904). It was then devised to grow immature or anomalous embryos, and defective seeds. Later, this method proved to be satisfactory for the study of embryo physiology, to produce hybrids of some plants too distantly related for natural seed and seedling development, and to promote germination of certain dormant seeds.

The objective of the work here reported was to obtain a method of germinating the seeds of *Comptonia peregrina* free of microorganisms and under known environmental conditions. This is a preliminary technique necessary for further work to be done to establish the effectiveness of nitrogen fixation within the root nodules. The method could also be used to study the process by which the seed plant becomes invaded by the nodular symbiont. An investigation of this symbiotic relation has been begun by the present writer.

DESCRIPTION OF THE SEED: The seeds are contained in a bur-like fruit about 1-2 cm in diameter, and contain about 8 nutlets. These are ellipsoid, blunt, 4-5 mm in length, olive brown, and lustrous. They consist of a thick bony exocarp, and an embryo 3 mm by 1.5 mm, without endosperm. Germination figures are not known to have been published.

The development of the seed was followed at the Cloquet Forest Research Center. During the 1960 growing season, the seed ripened during the last week of July. These nutlets are judged to be ripe when they have lost their watery or milky characteristics, and are firm, and fully developed. Seed quality was ascertained by cutting tests in the field. Areas producing high percentage of hollow or weeviled seed were avoided. It was found that many colonies actually have a fairly low percentage of filled, healthy seed.

METHOD: The bony exocarp or outer seed coat of the sweet fern seed is relatively impervious to moisture, and is difficult to remove in a dry condition. Two cellulose dissolving chemicals: concentrated sulfuric acid, and Cross-Devan's reagent (zinc chloride in twice its weight of concentrated hydrochloric acid) had no noticeable effect on this outer seed coat: neither did the usual hot water treatment, nor the prolonged use of stratification media, nor soaking in various organic solvents.

The embryos were most readily excised after soaking the seed in water for one or two days. The water should be changed at least twice daily. Since all empty seeds remain buoyant, these can be detected without further effort. Using a dissecting scope, the outer seed coats were cut or carefully slit with a sharp razor blade. With the aid of a dissecting needle, the embryos were removed and placed in a sterile petri plate of distilled water. The inner seed coat is composed of closely arranged, impervious cells. The water-resisting nature of this material prevented seed germination. In this study, the inner seed coat was removed with a sterile needle.

The sweet fern embryos were then treated for various periods of

time in commercial "chlorox" (sodium hypochlorite) diluted one to six with water. Bromine water proved to be excellent, because of its volatility, but difficult to prepare and use. The results showed that the chlorine solution was just as effective, and that immersion of 1 to 2 minutes caused no injury. No advantage was gained by washing the embryos with sterile water following treatment. The most satisfactory method of transferring the excised embryos was a firm bacteriological inoculation loop.

The medium utilized in this study was adapted from that suggested by Asen and Larson (1951). A ⅔% agar proved most desirable, since it was sufficiently firm to support the embryo, and still provide adequate moisture. Experiments showed that the roots also need the aeration available in the uppermost layer of the agar gel medium. Neither seedlings nor more mature plants could endure immersion in unaerated liquid culture media. Embryo culture tests indicated that 2% sucrose was more effective in promoting growth than 0.5% glucose. Therefore, the mixture used was made up by adding to one liter of distilled water: 20 grams of sucrose, 6.5 grams of agar, and 1.5 grams of the following mixture of mineral salts. This mixture consisted of: 10 grams of KCl, 2.3 grams of $CaSO_4$, 2.5 grams $MgSO_4$, 2.5 grams $Ca_3(PO_4)_2$, 2.5 grams $FePO_4$, and 2.0 grams KNO_3. Solution of the nutrients and dispersion of the agar were attained by stirring and gentle heating to boiling. Various types of containers have been used by others, but the 4-ounce bottles: 12 cm. tall, 6 cm. diameter, and having a mouth opening of 3 cm. were very satisfactory for culturing of *Comptonia* to the size desired. In filling the bottles, the usual precautions for bacteriological media were followed. Non-absorbent cotton plugs were used to stopper the containers. The medium was sterilized by autoclaving at 15 pounds pressure for 20 minutes. The agar medium solidifies when cool.

The standard bacteriological procedures were followed in preparing the cultures. This included: flame sterilization of the instruments, holding open bottle horizontal, flaming the openings of test tubes, and bottles. The number of embryos to culture in one bottle is, of course, dependent on the container size. A maximum of 5 embryos could be handled efficiently in the 4-ounce bottles used. It should be pointed out, however, that the more embryos placed in the bottle, the greater is the chance for contamination.

RESULTS: Satisfactory seedling development was obtained in a room with direct August sunlight, but care was taken to prevent the rise in temperature of the bottles to levels high enough to cause injury. The embryo culture tests showed that besides the high light intensity requirement, the seedlings also had increased growth at higher room temperatures of 80° to 90° Fahrenheit. With these temperatures and direct sunlight, good germination of 80% of the uninfected embryos was obtained in two to three weeks. The first true leaves expanded about a week later.

At the end of the growing season, beginning on September 21st, the use of additional artificial light to lengthen the photoperiod to 16 hours did not prevent the cessation of growth of the seedling or the more mature plant. Further studies will be made to ascertain the effect of controlling the temperature, as well as the light in eliminating this dormancy.

SUMMARY: No previous work on the propagation of *Comptonia peregrina* nor the variety *asplenifolia* of this North American genus of the family Myricaceae has been found in the literature. Field studies in Carlton County in Minnesota and Douglas County in Wisconsin in the spring and early summer of 1959, 1960, and 1961 have revealed only an occasional seedling. Natural dispersal of this shrub in this northwesterly portion of its range would seem at present to be occurring only slowly through gradual extension of rhizomes. The embryo excision method overcomes the dormancy of the sweet fern seeds which as has been shown is not conditioned by physiological reactions in the embryo, but rather by the inhibiting effects of the seed coats. By excising directly from the fruits after the seeds are fully developed, using only high quality seed, and maintaining strict aseptic conditions in the handling of media and embryos, the number of contaminated cultures are negligible. The method devised in this study appears to be a means of accelerating growth studies of the seedlings. In addition, it ensures the specificity and exact experimental conditions necessary to ascertain whether nitrogen fixation does indeed occur.

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