Maintaining *Bremia lactucae* on Washed Seedlings of *Lactuca sativa* in Deep Petri Dishes

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ABSTRACT

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Attempts to culture *Bremia lactucae*, an obligate parasite, on living host tissue in closed dishes were not successful because of contamination by other microorganisms during either germination or inoculation. Contamination was avoided with a new technique similar to growing bean sprouts. About 60 lettuce seeds were sown on moist filter paper in a 10-cm petri dish and incubated at room temperature. When the cotyledons had emerged but were not unfolded (5–7 days later), the seedlings were transferred to a 10-cm deep petri dish, washed several times with tap water to remove seed coats, and incubated at 14 C in an illuminated incubator (3,000 lux, 12-hr photoperiod). The seedlings were then washed daily with two to three changes of tap water. When the cotyledons had expanded, they were sprayed to runoff with a suspension of sporangia of *B. lactucae* in distilled water (1×10^5 sporangia per milliliter), and the daily washings were resumed the next day. The fungus sporulated on both surfaces of the cotyledons in 10–14 days. This technique was also used with detached cotyledons: the two cotyledons and about 1 cm of hypocotyl were removed from seedlings, placed in 125-ml Erlenmeyer flasks, inoculated, incubated, and washed as for seedlings until the fungus sporulated.

Additional key words: downy mildew.

Bremia lactucae Regel, the causal agent of downy mildew of lettuce (*Lactuca sativa* L.), is an obligate parasite, requiring living host tissue in order to grow. *B. lactucae* infects lettuce at temperatures between 2 and 25 C and relative humidity near 100% (5). The fungus sporulates at relative humidity of 90–100% (5). Within these limits, several methods for growing *B. lactucae* have been developed (4), which can be classified into two groups. In the first type of procedure, entire plants are grown to various stages of maturity, inoculated, maintained at high humidity to allow infection by the fungus, incubated for a set period under dry conditions, and then returned to high humidity to induce sporulation. One variation of this procedure was used by Sequeira and Raffray (7), who grew seedlings to the cotyledon stage at 25 C (light intensity 2,000 ft-c) and incubated them in a dark dew chamber at 18 C for 24 hr after inoculation. The same dew chamber was used to induce sporulation 1 wk later. Another variation of this procedure was used by Jones and Leeper (3), who grew seedlings to the three- to four-leaf stage at 18 C (light intensity 500 ft-c) and covered the pots with transparent plastic for inoculation and induction of sporulation.

In the second type of procedure, most commonly used in Europe, unpotted seedlings, detached leaves or cotyledons, or excised leaf disks are placed in a humid environment for maintenance, inoculation, and incubation of the fungus. Variations of this procedure have been used for maintenance of *B. lactucae* by Dickinson and Crute for seedlings (2), Channon et al for detached cotyledons (1), and Rodenburg for leaf disks (6).

In the first procedure, because relative humidity normally is low, the fungus does not sporulate until the humidity is raised. In the second procedure, because relative humidity normally is high, sporulation occurs when the fungus reaches a minimum density in the lettuce tissue. Thus, sporulation is easier to control in the first than in the second procedure.

Each procedure for culturing *B. lactucae* has its advantages. The first procedure allows growth of plants outside the humid environment and prevents sporulation until desired. However, absolute separation of isolates is difficult with this method. Jones and Leeper (3) used two separate growth chambers to prevent the mixing of races. The second procedure uses a closed system, which is an advantage if a number of clonal isolates or races must be maintained free of contamination. However, sporulation is harder to control and contamination with other microorganisms can be a problem. We used both procedures, but contamination by other fungi and bacteria was frequent and was especially severe when field-collected sporangia were used as inoculum. This difficulty led to the development of the method of growing *B. lactucae* reported herein, in which unwanted microorganisms are reduced by washing the lettuce seedlings in a manner similar to that used in growing bean sprouts.

MATERIALS AND METHODS

Lettuce seeds (cv. Ithaca from Harris Seed Company, Rochester, NY 14624) were placed on moist filter paper in 10-cm glass petri dishes and incubated at room temperature and lighting for 5 days. At this time, the cotyledons had emerged but had not completely unfolded. The seedlings then were transferred to a deep petri dish (100×80 mm storage dish number 3250, Corning Glass Works, Corning, NY 14830) and washed several times with tap water to remove most of the seed coats. The dishes were maintained in an illuminated incubator (14 C; 3,000 lux; 12-hr photoperiod). All subsequent incubation of seedlings or leaves was done in this incubator under these conditions. The seedlings were washed daily by filling the dish with tap water and then draining off the excess water. This washing procedure was continued until the cotyledons had completely expanded.

Isolates of *B. lactucae* were obtained from commercial lettuce fields in Oswego County, NY. Leaves with typical symptoms of downy mildew were collected in the field, placed in plastic bags, and transported to the laboratory the same day. The leaves were washed briefly in tap water and then placed in glass casseroles (with a small amount of water in the bottom) and incubated until sporulation occurred.

To remove sporangia, the sporulating lesions were shaken in a flask with distilled water, and the suspension was filtered through a single layer of cheesecloth. During routine maintenance, sporangia were collected from sporulating seedlings in a similar manner. The concentration of the resulting suspension was determined by counting the number of sporangia with a hemacytometer and adjusted to 10^5 sporangia per milliliter. If the concentration was greater than 10^5 sporangia per milliliter, distilled water was added. If the concentration was less than 10^5 sporangia per milliliter, the suspension was centrifuged at 10,500 *g* for 10 min, and pelleted sporangia were resuspended in an amount of distilled water calculated to yield a final concentration of 10^5 sporangia per

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milliliter of water.

Washed lettuce seedlings with fully expanded cotyledons were sprayed to runoff with the sporangial suspension with a Preval sprayer (Precision Valve Corp., Yonkers, NY 10702). The daily washing was interrupted for 1 day to allow the sporangia to germinate and infect the cotyledons of the lettuce seedlings. The washing was resumed the following day and continued until sporulation occurred.

A modification of the above technique used detached portions of lettuce seedlings grown in the growth chamber or greenhouse as a substrate for the fungus. Lettuce seeds were sown on sterile vermiculite and subirrigated. After 5 days, the cotyledons had expanded enough to allow inoculation. The upper portion of the seedlings (both cotyledons and about 1 cm of hypocotyl) was detached with a razor blade or scissors, placed in Erlenmeyer flasks, and inoculated with a suspension of sporangia. The flasks were closed with rubber stoppers or plastic film and placed in the illuminated incubator. The detached cotyledons were washed daily until the fungus sporulated.

RESULTS

Lettuce seedlings that germinated in the deep petri dishes became entangled and produced a seedling mat that could be treated as a single unit during the washing procedure (Fig. 1A). This mat was ready for inoculation about 10 days after lettuce seed

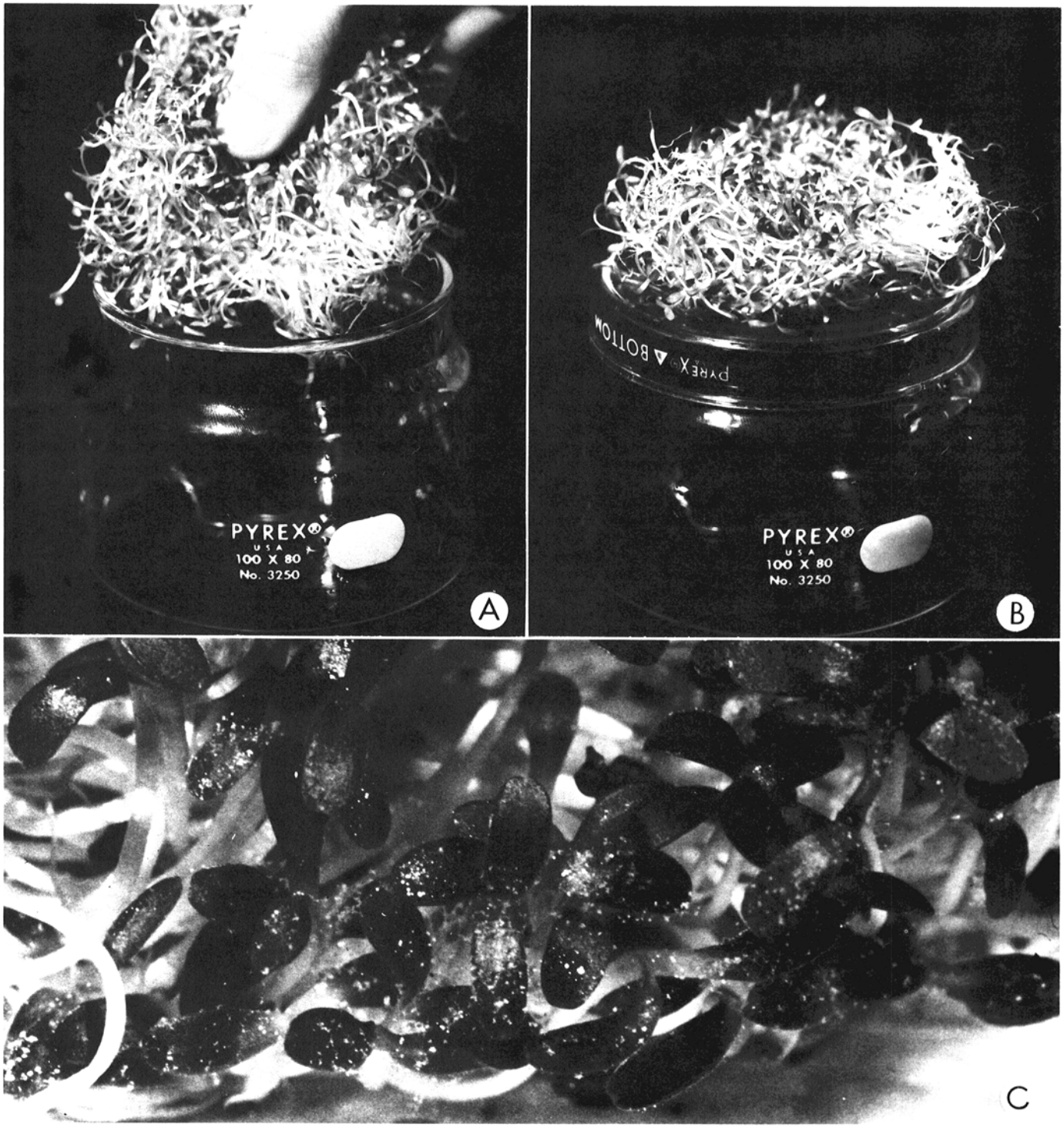

Fig. 1. Lettuce seedlings during incubation. **A,** Seedlings entwined to form a seedling mat that can be handled as a single unit. **B,** Seedlings ready to be inoculated. **C,** *Bremia lactucae* sporangia on lettuce cotyledons.

was sown on the moist filter paper (Fig. 1B). Detached cotyledons also formed mats in the Erlenmeyer flasks, but the adventitious roots that developed were much shorter, and these mats were easily separated.

When field-collected lettuce leaves with lesions caused by *B. lactucae* were incubated to produce sporangia, the time from collection to sporulation varied, generally ranging from 3 to 7 days. The sporangia harvested from these lesions consisted of a mixture of old and fresh sporangia, and when this mixture was used to inoculate the lettuce seedlings, it did not provide a concentration of 10^5 viable sporangia per milliliter. Because the concentration of viable sporangia differed with each collection, the time from inoculation to subsequent sporulation also varied, generally ranging from 10 to 20 days, because the fungus sporulated only after the mycelium reached a minimum density in the tissue (2). Thus, spore concentration was inversely related to length of time to sporulation.

When fresh sporangia from a seedling mat were used as a source of inoculum, most of the sporangia were viable, and the time from inoculation of another seedling mat to subsequent sporulation was 10 days (Fig. 1C).

DISCUSSION

Growing *B. lactucae* in the laboratory always has been hampered by the need for living lettuce tissue. Consequently, this fungus, along with many other obligate parasites, has not been studied as extensively as plant pathogens that can be grown more easily. Our technique for growing *B. lactucae* on either lettuce seedlings or detached cotyledons in glass culture dishes has several advantages over previously described procedures. The lettuce tissue is easily and rapidly produced, and although we occasionally used growth chambers to grow seedlings, no special facilities actually are required. The washing removes or reduces most of the unwanted saprophytes and keeps the lettuce tissue relatively healthy. The seedling mats that develop in the dishes or flasks are maintained without any supportive substrate or nutrient solutions. These mats can be handled as individual units when the seedlings have entwined, allowing large numbers of seedlings to be grown (and large numbers of sporangia to be produced) without handling individual plants.

A major advantage of the new technique is that it provides a closed system with much less opportunity for cross-contamination among clonal isolates or races of *B. lactucae* than systems previously described by Sequeira and Raffray (7) and Jones and Leeper (3). Studies on the genetics of *B. lactucae* require reliable separation of isolates. While the method described by Dickinson and Crute (2) does restrict cross-contamination, we could not maintain cultures using their method because of contamination by other microorganisms.

The washed seedling technique has made possible extensive studies on the occurrence and frequency of different races of *B. lactucae* and less extensive studies on the occurrence of its mating types. The technique could also provide large quantities of sporangia for studies on oospore formation and the role of these oospores in the pathogenesis of the fungus on lettuce.

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