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# Development of a Defined Medium for Growth of *Cercospora rosicola* Passerini

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A simple synthetic liquid medium containing a single amino acid, glucose, salts, trace metals, and thiamine was developed for cultivation of *Cercospora rosicola* Passerini. Thiamine was shown to be important to growth. Culture of *C. rosicola* Passerini in a chemically defined medium makes possible studies of (+)-abscisic acid biosynthesis and regulation.

*Cercospora rosicola* Passerini is the only mold reported to produce the secondary metabolite (+)-abscisic acid (ABA) (2). ABA is an important plant hormone involved not only in growth inhibition but also in many aspects of growth and development in plants from seed and bud dormancy to maturity, senescence, and abscision. The intermediate steps of the biosynthetic pathway between mevalonate and ABA are not known, and no enzymes of that system have been isolated. The fungus *C. rosicola* offers a new and convenient means of studying the biosynthesis and regulation of ABA.

Little information is available on the culture of *C. rosicola*. Assante et al. (2) reported that the fungus produced ABA when grown on agar containing potato, yeast, malt, and oatmeal extracts but did not produce ABA when grown on agar containing peptone and glucose or Sabouraud, Czapek, and nutrient formulas. No information was given about the relative growth of *C. rosicola* on these media. We found the growth of *C. rosicola* to be erratic and not predictable in liquid shake culture with complex natural extracts. This report describes the development of a chemically defined medium that supports growth of *C. rosicola* in liquid shake culture.

*C. rosicola* (strain 138.35) cultures were obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. Primary slants and working petri dish cultures were maintained on potato-dextrose-agar.

L-Amino acids, vitamins, and inorganic salts were of the highest purity and were purchased from commercial sources. Reverse osmosis-deionized water (Millipore Mulli-Q System) was used for all procedures.

A synthetic medium was formulated by inclusion of most of the components of potato (1, 5). This synthetic medium contained (per milliliter): 20 mg of glucose, 3 mg of fructose, 3 mg of sucrose, 0.20 mg of $MgSO_4$, 0.8 mg of $KH_2PO_4$, 0.1 mg of $CaCl_2 \cdot H_2O$, 0.5 mg of KCl, 0.001 mg of thiamine, 0.008 mg of riboflavin, 0.003 mg of niacin, 0.04 mg of ascorbic acid, 0.50 mg of aspartic acid, 0.41 mg of glutamic acid, 0.24 mg of leucine, 0.20 mg of arginine, 0.19 mg of lysine, 0.19 mg of valine, 0.17 mg of serine, 0.18 mg of alanine, 0.16 mg of phenylalanine, 0.15 mg of isoleucine, 0.15 mg of threonine, 0.15 mg of glycine, 0.15 mg of proline, 0.11 mg of tyrosine, 0.06 mg of histidine, 0.05 mg of methionine, and 0.02 mg of cysteine. Also, each liter of test medium contained 1 ml of trace metals (0.05 g of $FeSO_4 \cdot 7H_2O$, 0.033 g of $MnCl_2$, 0.25 g of $ZnSO_4$, 0.40 g of $CuSO_4 \cdot 4H_2O$, and 0.00005 g of $H_3BO_3$ in 100 ml of water). Except for glucose, inorganic salts, and trace metals, the above medium was varied to find a combination and concentration of compounds that would support the most growth of *C. rosicola*. Medium with one, two, or three of the above amino acids contained 1 mg, 0.5 mg of each, or 0.33 mg of each, respectively, per ml. Medium with other single nitrogen sources, i.e., glutamine, asparagine, urea, $NH_4NO_3$, $CH_3COONH_4$, or monosodium glutamate (MSG), contained an amount equivalent to 0.23 mg of nitrogen per ml. Potato broth contained 20 mg of glucose and 4 mg of dehydrated potato extract per ml. The pH values of the media were adjusted to 6.5 with 0.1 N HCl or NaOH. All media were sterilized by autoclave, 15 min at 15 lb/in$^2$, except where stated otherwise. Millex single-use filter units (Millipore Corp.) were used for sterilization by filtration. Mycelia, cut from the surface of potato-dextrose-agar cultures, were blended for 30 s with 100 ml of sterile water in a Waring blender. Sterile test media (100 ml) in 500-ml Erlenmeyer flasks were inoculated with 2 ml of the blended mycelia. All treatments in duplicate liquid cultures were incubated on reciprocating shakers at 24 to 28°C for 1 to 4 weeks under continuous fluorescent lighting. Liquid cultures were filtered through

tared filter paper (Whatman no. 3) at reduced pressure. Filter disks and mycelia in tared disposable petri dishes were frozen in dry ice, freeze-dried, and weighed.

Potato extract medium and the synthetic potato medium were compared for growth of *C. rosicola*. The synthetic medium caused a slight growth lag but supported more growth than did the potato extract medium after 21 days (Fig. 1).

In subsequent tests, different components were deleted from the synthetic medium to determine requirements for growth of *C. rosicola*. Deletion of fructose and sucrose did not affect growth. Growth increased about 10-fold when cysteine was omitted. Cysteine is reported to be toxic to some fungi (7). Growth was not affected by the omission of the nine amino acids present in the smallest concentrations (alanine through methionine). The remaining seven amino acids were tested together and in groups of two or three and singly for growth of *C. rosicola*. Glutamine, asparagine, urea, MSG, ammonium nitrate, ammonium acetate, and potato extract were also tested.

Urea supported growth of up to 5 mg/ml when autoclaved, but did not support growth when sterilized by filtration. Urea breaks down to ammonia and biuret or $CO_2$ when heated (3). Aside from a mixture of arginine, lysine, and valine,

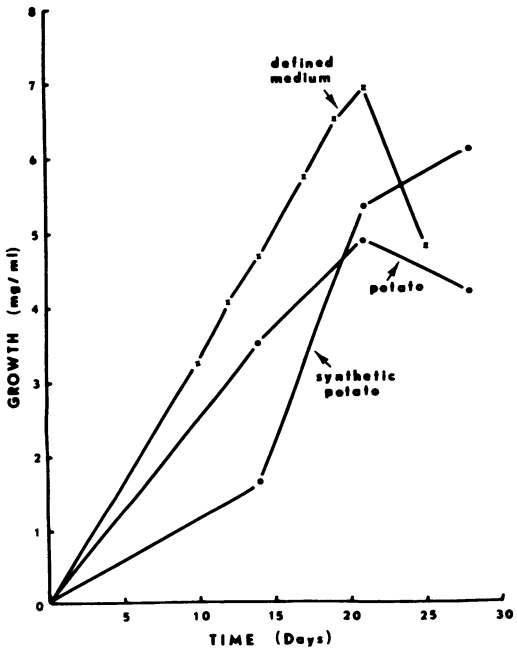


FIG. 1. *Comparison of potato extract, synthetic potato medium, and the chemically defined medium (see text) for growth of C. rosicola in liquid shake culture.*

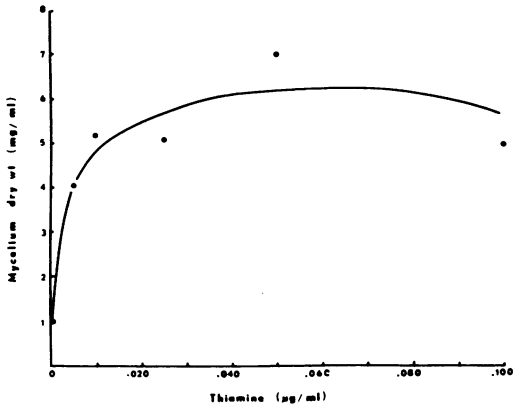


FIG. 2. *Growth of C. rosicola with different concentrations of thiamine in the medium.*

combinations of amino acids containing arginine supported growth of less than 1 mg/ml. Most combinations of amino acids, except for the same arginine-lysine-valine mixture, did not support growth as well as some of the single amino acids. Asparagine, MSG, glutamic acid, glycine, glutamine, and aspartic acid, respectively, supported 5.1, 4.4, 3.1, 3.8, 3.6, and 3.3 mg of mycelia per ml. Leucine and serine supported 1.7 and 1.3 mg of growth per ml. Arginine, valine, and lysine singly supported less than 1 mg of growth per ml. Growth in ammonium nitrate was 2.6 mg/ml, and ammonium acetate did not support growth. Growth in potato extract was 2.1 mg/ml and proved to be an intermediate to poor nutrient source. Browning may be responsible in part for the poor and erratic growth with the potato extract and some combinations of amino acids. Increasing the levels of glutamic acid or MSG above 3 mg/ml did not enhance growth. Glutamic acid or MSG was used as the sole source of nitrogen for further testing of the medium.

Combinations of thiamine, niacin, riboflavin, and ascorbic acid were tested for growth of *C. rosicola* with glutamic acid as the nitrogen source. Thiamine with and without niacin, riboflavin, and ascorbic acid supported 2 to 3 mg of growth per ml. Ascorbic acid may affect growth somewhat through its effect on the oxidation-reduction potential. Growth was reduced to less than 0.2 to 0.5 mg/ml when thiamine was omitted. Levels of thiamine were reduced until growth was proportional to the supply of thiamine (MSG was the nitrogen source). The lowest concentration of thiamine giving maximum growth of 7 mg/ml was 0.05 µg/ml, and growth was proportional to the amount of thiamine below this level (Fig. 2).

Frequently, a growth factor can be exchanged for a precursor constituting part of the growth

TABLE 1. *Growth response of C. rosicola to thiamine, cytosine, methyl cytosine, and pyrithiamine in the defined medium*[a]

| Medium component | Concn (μg/ml) | Sterilization method | Mycelial dry wt (mg/ml) |
|---|---|---|---|
| Control, no thiamine | 0 | Autoclaving | 0.91 |
| Thiamine | 100 | Autoclaving | 4.74 |
| Thiamine | 100 | Filtration | 5.40 |
| Thiamine | 50 | Autoclaving | 7.04 |
| Cytosine | 100 | Filtration | 2.78 |
| 5-Methyl cytosine | 100 | Filtration | 0.16 |
| Pyrithiamine | 100 | Filtration | 1.38 |

[a] Values represent the mean of duplicate cultures.

factor molecule (4, 6). Cytosine and 5-methyl cytosine were substituted for thiamine to determine whether the pyrimidine moiety could be used to fill the thiamine requirement. Cytosine supported half as much growth as thiamine, and 5-methyl cytosine inhibited growth (Table 1). Pyrithiamine is usually inhibitory to fungi that require intact thiamine and not inhibitory to fungi that synthesize thiamine (4). Slightly more growth than the control without thiamine was obtained when pyrithiamine was substituted for thiamine. Autoclaved thiamine supported slightly more growth than did filter-sterilized thiamine. *C. rosicola* appears to have a thiamine requirement, but further testing is required to determine whether the fungus is auxotrophic for thiamine precursors or the intact thiamine molecule. The small amount of growth obtained without thiamine may be due to trace impurities in the MSG or glutamic acid, because both are fermentation products. When inoculum from 7-day-old liquid seed cultures containing thiamine was used in place of surface potato-dextrose-agar cultures, growth was not affected by the presence of thiamine. Mycelial weights of 2.8 and 2.6 mg/ml were obtained after 1 week of culture without thamine and with 1.0 μg of thiamine per ml, respectively. Fungi with very small thiamine requirements are sometimes able to synthesize thiamine once growth is initiated (4), or the mycelia may contain enough thiamine to support growth.

Amounts of phosphate above 0.8 mg/ml did not enhance growth of *C. rosicola*. The amounts of salts and trace metals used in the synthetic potato medium were used throughout the study. Other salts and trace metals were not investigated.

The final defined medium was simple and easy to prepare. In addition to 1 ml of the trace metals mixture described above, the defined medium contained (per milliliter): 20.0 g of glucose, 0.2 g of $MgSO_4$, 0.5 g of KCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.8 g of $KH_2PO_4$, 0.001 g of thiamine, and 3.0 g of MSG. The growth rate of *C. rosicola* in this medium was faster, and mycelial dry weight reached a maximum of 6.9 mg/ml compared with 5.3 and 4.9 mg/ml for the same period in complete synthetic potato and the potato extract, respectively (Fig. 1). There was no lag phase with the defined medium. MSG was a more preferable nitrogen source than urea, asparagine, and glutamic acid because it is more stable and the pH is more constant during autoclaving. The pH of the medium was about 6 before autoclaving (without adjustment) and about 6.5 after autoclaving. Little browning took place.

Although the object of this study was to formulate a synthetic defined medium for the growth of *C. rosicola* for subsequent study of ABA biosynthesis and regulation, considerable information on ABA production was also obtained. Different nutrient components of the medium have profound effects on ABA biosynthesis, and these results will be reported separately. (Norman, Maier, and Echols, submitted for publication). The development of a chemically defined liquid medium opens the way to controlled studies of ABA biosynthesis and regulation with this fungus.

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