Victorin Production Stimulated by Oat Flakes

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Accepted for publication 13 January 1981.

ABSTRACT

Wheeler, H., and Gantz, D. 1981. Victorin production stimulated by oat flakes. Phytopathology 71:853-854.

Fries' solution, often supplemented with yeast extract, has served for many years as a standard medium for production of victorin by *Helminthosporium victoriae* and of selective pathotoxins by several other fungal pathogens. Adding oat flakes to Fries' solution eliminated the need for yeast extract and greatly increased toxin titer. In media containing flakes, maximum toxin titers were achieved in 10–12 days instead of 3–4 wk, and residual dry weights of culture fluids were reduced by 30–50%.

Fries' solution was first used as a medium for victorin production by *Helminthosporium victoriae* Meehan and Murphy after an extensive search for a chemically defined substrate that would provide high yields of toxin (1). With this medium, highest yields of victorin were obtained from stationary cultures grown for 15–25 days on shallow layers (20–30 ml) of medium in small (125-ml) Erlenmeyer flasks. Batches of crude culture filtrates produced under these conditions caused an average 50% inhibition in root growth assays when diluted 2×10^6-fold or, in exceptional cases, when diluted 2×10^7-fold. On equally shallow layers of medium in 2-L flasks, toxin titers were 10- to 100-fold lower (1).

Since its introduction, Fries' medium, often supplemented with 0.1–1.0 g of yeast extract per liter, has been used in many laboratories, not only for victorin production but also for production of several other selective pathotoxins (2–4). Extremely high yields of victorin from stationary cultures have been reported; in one case, a crude culture filtrate was active (50% inhibition in the root growth assay) when diluted 2×10^8-fold (2). However, most such crude preparations have been much less active, giving 50% inhibition when diluted 10^4–10^5-fold (1,4,5).

Recently, victorin titers comparable to average yields from stationary cultures have been obtained in shake cultures inoculated with fragments of blended mycelium (4). With this method, maximum yields of toxin were obtained in 6 days rather than 3–4 wk, and residual dry weights of culture fluids were less than half those found with stationary cultures. Despite these advantages, maximum victorin titers obtained with shake cultures (50% root growth inhibition with culture fluids diluted 10^5-fold) were less than 1% of the highest titers reported from stationary cultures (2).

A survey of attempts over the past 30 yr to produce victorin in the senior author's laboratory showed that unexplained and very large (up to three orders of magnitude) variations in toxin titers were common. This survey also revealed that very high toxin titers had been obtained only when spores and fragments of mycelium scraped from cultures growing on oatmeal agar were transferred directly into small volumes of Fries' medium. Conversely, in a few cases when production flasks were seeded with well-washed spores, toxin yields were very low. These findings suggested that oatmeal or some other material, introduced when production flasks were seeded, might be essential for high yields of victorin, and led to tests with media supplemented with oat flakes and grains.

MATERIALS AND METHODS

All media for victorin production contained Fries' salts (5 g of ammonium tartrate, 1 g of NH_4NO_3, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl and 0.1 g of $CaCl_2$ per liter) supplemented with sucrose, yeast extract, oat flakes, or oat grains. Several different commercial brands of rolled oats (flakes) all gave similar results. Results with supplements of oat grains of cultivars susceptible to Victoria blight (Park, Victorgrain 48-93) did not differ from those with grains from resistant cultivars (Compact, Dubois).

Unless otherwise specified, 125-ml Erlenmeyer production flasks containing 30 ml of medium were used. These flasks were seeded with spores scraped from the surface of *H. victoriae* cultures, isolate HV1 (6), grown for 14 days on oatmeal agar slants (care was taken not to transfer bits of the agar medium). After seeding, production flasks were left undisturbed on laboratory benches under fluctuating room temperature and light until samples were taken for determination of residual dry weights of culture fluids and for assays by the root growth test (1) with the oat cultivar Park. Samples consisted of the pooled contents of three flasks expressed through four layers of cheesecloth. With media containing oat flakes or oat grains, samples were taken daily beginning 8 days after flasks were seeded. With other media, samples were taken at 2-day intervals beginning 15 days after seeding. In all cases, sampling was continued until two consecutive samples failed to show an increase of more than 5% in root growth inhibition with culture fluids diluted 10^6-fold.

RESULTS AND DISCUSSION

Data from a series of preliminary tests (Table 1) indicated that adding 0.5 g/L of yeast extract to media containing 30 g of sucrose per liter increased toxin titer 10-fold, greatly reduced residual dry weight of culture fluids, and slightly reduced the time required to attain maximum toxin titer. These effects of yeast extract were very similar to those reported with blended mycelium in shake cultures (4). Adding 15 or 25 g of oat flakes per liter to media containing 30 g of sucrose increased toxin titers 100-fold and reduced residual dry weights and times required to attain maximum toxin titers by 50% (Table 1). Yeast extract added to media containing oat flakes did not increase toxin titers and had the adverse effect of increasing residual dry weights somewhat. On media supplemented with oat grains, reductions in times required to attain maximum toxin titers and in residual dry weights were similar to those obtained with oat flakes. Increases in toxin titers with oat grains, however, were small compared with those with oat flakes and were not independent of added yeast extract.

Because the highest toxin titers and the lowest residual dry weights were obtained with 15 g of sucrose and 15 g of oat flakes per liter (Table 1), this combination was used to test the effects of flask size and volume of medium on victorin production. With volumes of medium adjusted to give approximately equal depths in all flasks, variations in toxin titer were small compared with those previously reported without added oat flakes (1) and were not correlated with flask size or volume of medium (Table 2). Three large (20–30 L) batches of victorin have been produced with mixtures of flask sizes, with the same volumes of the medium used to obtain the data in Table 2. All three batches inhibited root growth by more than 50% when diluted 10^7-fold, and residual dry

0031-949X/81/08085302/$3.00/0

TABLE 1. Effects on victorin production of sucrose, yeast extract, oat flakes, and oat grains added to solutions of Fries' salts

Materials added (g/L)				Growth		Root growth inhibition (%) in concentrations of:		
Sucrose	Yeast extract	Oat flakes	Oat grains	period[a] (days)	RDW[b] (mg/ml)	10^{-5}	10^{-6}	10^{-7}
30	0	0	0	23	23.8	48	8	0
30	0.5	0	0	19	12.8	82	48	0
30	0	25	0	11	11.0	96	83	42
30	0.5	25	0	11	12.6	98	81	40
30	0	15	0	12	10.4	94	86	48
30	0.5	15	0	10	13.2	91	78	25
15	0	15	0	11	6.6	98	88	65
15	0.5	15	0	11	9.7	97	84	58
30	0	0	15	13	10.6	65	22	0
30	0.5	0	15	12	11.4	89	56	12

[a] Time required to attain maximum toxin titer.
[b] Residual dry weight of culture fluid.

TABLE 2. Effect of flask size and volume of medium on victorin production after 12 days in Fries' medium containing 15 g of sucrose plus 15 g of oat flakes per liter

Flask size (ml)	Volume of medium (ml)	RDW[a] (mg/ml)	Root growth inhibition (%) in concentrations of: 10^{-5}	10^{-6}	10^{-7}
125	30	6.1	93	80	51
250	45	6.0	98	86	79
500	75	7.0	98	87	52
1,000	125	9.2	91	67	42
2,800	250	6.4	98	77	56

[a] Residual dry weight of culture fluid.

weights of culture fluids ranged from 5.2 to 6.4 mg/ml. These results and those in Table 2 indicate that the large variations in toxin titer and low yields in large flasks that have occurred with unsupplemented Fries' medium are the result of variations in the concentration of oatmeal or some other stimulatory material introduced in the process of seeding production flasks.

Freshly expressed fluids from cultures grown on Fries' medium supplemented with oat flakes were cloudy but could be clarified by holding overnight at 4 C and then filtering or centrifuging to remove sedimented material. Such clarification reduced residual dry weights by 10–15%, with no reduction in toxin titer.

One 20-L batch of clarified filtrate was mixed with an equal volume of acetone to precipitate macromolecules and to provide a preparation that could be compared with similar preparations from shake cultures in terms of specific activity. After the precipitate and the acetone were removed, this preparation had a residual dry weight of 4.7 mg/ml and a specific activity, defined as the amount of toxin required to produce 50% inhibition in root growth assays, of 0.47 ng/ml, compared with a residual dry weight of 4.5 mg/ml and a specific activity of 45 ng/ml for victorin produced in shake culture (4). Although victorin production in shake culture saves time (5–6 days rather than 10–12 days), this advantage appears to be more than offset by the 100-fold higher toxin titers obtained in still culture on media containing oat flakes.

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