Regulation of nitrate reductase, soluble and protein nitrogen by lawsone in *Helminthosporium oryzae* Breda de Haan[1]

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Summary. The mechanism of antifungal action of 2-hydroxy-1,4-naphthoquinone (lawsone) isolated from leaves of *Lawsonia inermis* was studied. It was found to inhibit nitrate reductase activity in vivo and in vitro. The concentration which inhibited in vivo enzyme activity also reduced ethanol-soluble and ethanol-insoluble nitrogen (protein nitrogen) content in the test organism, *Helminthosporium oryzae.*

Lawsone (2-hydroxy-1,4-naphthoquinone) was isolated from the leaves of *Lawsonia inermis* as the sole antifungal antibiotic[4]. Although substituted α-naphthoquinones are reported to inhibit various enzyme systems in fungi[5–9], there appears to be no report describing the effects of these compounds on nitrate reductase activity and on soluble and protein nitrogen content. Inhibition of an enzyme such as nitrate reductase must have an effect on the input of reduced nitrogen, and consequently on the level of amino acids and proteins. Such effects could result in the inhibition of overall growth of the fungus. In the present paper, therefore, the effect of 650 ppm lawsone, (LD-50, sublethal concentration at which 50% growth of the organism is inhibited[10]) on nitrate reductase activity, soluble and protein nitrogen content of the fungal cells have been investigated and the possible association of such effects with the mechanism responsible for killing the fungus discussed.

Experimental procedure and results. The culture of *Helminthosporium oryzae* Breda de Haan was obtained from the Mycology Division, I.A.R.I., New Delhi. The fungus was grown in Czapek's liquid medium. Cultures were grown with and without lawsone (650 ppm), and harvested at different intervals (from 4 to 8 days). In vivo nitrate reductase activity was assayed according to the procedure described earlier by measuring the total amount of nitrite produced[11]. As the in vivo enzyme activity was found to be maximal on the 7th day, it was extracted on that day for in vitro studies. The method of extraction followed was that used by Choudary and Rao[12]. The assay mixture contained 1 ml of the enzyme extract, 0.02 M KNO_3, 0.02 mM NADH, 0.2 M potassium phosphate buffer (pH 7.4), and lawsone solution (at varying concentrations) in a total volume of 7.5 ml. The mixture was incubated for 1 h and NO_2 produced was estimated by the procedure described[11]. In another set L-cysteine was added to give a final concentration of 5×10^{-3} M in all the control and treatment sets.

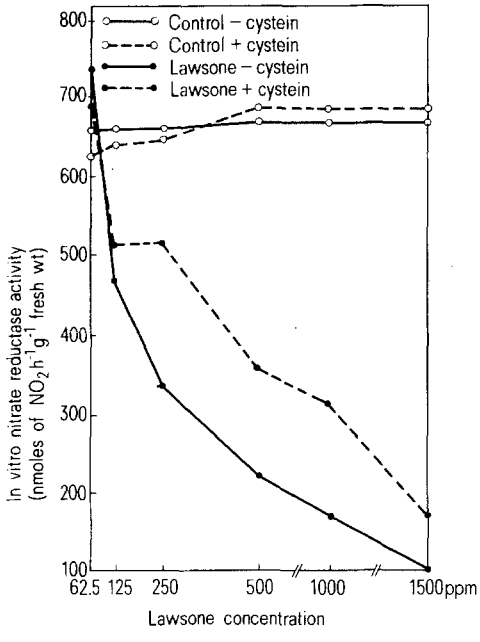

Fig. 1. Effect of different concentrations of lawsone on in vitro nitrate reductase activity in presence and absence of cysteine.

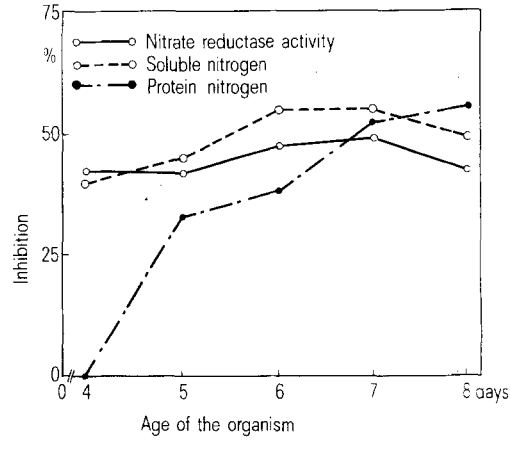

Fig. 2. Effect of lawsone on in vivo nitrate reductase activity and on soluble and protein nitrogen in the test organism.

Effect pf 650 ppm lawsone on ethanol-soluble and protein nitrogen and their percent inhibition in the test organism

Organism age in days	Control mg g^{-1} dry wt				Treatment mg g^{-1} dry wt				% Inhibition			
	Soluble nitrogen	Protein nitrogen	Total nitrogen	Protein	Soluble nitrogen	Protein nitrogen	Total nitrogen	Protein	Soluble nitrogen	Protein nitrogen	Total nitrogen	Protein
4	20	26	46	162.5	12	26	38	156.3	40	0	17.39	0
5	21	30	51	187.5	11.5	20	31.5	125.0	45.23	33.33	38.23	33.33
6	26	31	57	193.7	11.45	19	29.45	118.8	55.96	38.70	48.33	38.70
7	25	31	56	193.7	11	14.5	25.5	92.74	56	53.33	54.46	53.33
8	25	28	53	175.0	12.75	12	24.75	75.11	51	57.14	54.24	57.08

Lawsone and the other constituents of the assay mixture were as usual. Nitrite production was estimated as before. The nitrogen content, both ethanol-soluble and ethanol-insoluble (protein) was determined using the method described earlier[13]. A PR-2 international refrigerated centrifuge and a DU_2 spectrophotometer were used as and when needed.

In the control the fungus showed a gradual increase in the enzyme activity up to only the 6th–7th day of growth. Lawsone at different sampling times inhibited in vivo enzyme activity by 42–50%. As shown in figure 1, inhibition of in vitro nitrate reductase activity increased with increasing concentrations of lawsone; the maximum inhibition (70%) was obtained at 1500 ppm. The lowest concentration of lawsone (62.5 ppm) used stimulated nitrate reductase activity by 13%. It is obvious from figure 1 that lawsone action on the enzyme in the presence of L-cysteine showed a similar pattern to that in experiments without L-cysteine (figure 1) and that the inhibition of enzyme activity at higher concentrations of lawsone was not prevented by cysteine. The table shows the effect of lawsone on ethanol-soluble and protein nitrogen content. While the amount of soluble nitrogen was adversely affected from the beginning of the growth period, the protein content was affected only after the 4th day of growth.

Discussion. The present study showed that on day 4 lawsone inhibited in vivo enzyme activity by 42.3% and at the same time soluble nitrogen content was also reduced by 40% approximately. Over the 5-day-periods (day 4–day 8) of growth there was a parallel decrease in the inhibition of nitrate reductase activity and in soluble nitrogen content (figure 2). This suggests that there exists a correlation between nitrate reductase activity and soluble nitrogen. No such correlation, however, was observed between inhibition of nitrate reductase and reduction of protein nitrogen content (figure 2). It would, therefore, appear that the effect of lawsone on protein synthesis was not entirely through its action on nitrate reductase. This indicates that lawsone affects the protein synthesis, at least partly, through its action on some points other than nitrate reductase (an alternative route). Although our data are not sufficient to indicate a definite mechanism of inhibition, it might be similar to the inhibition of papain, an SH containing enzyme, caused by 2-methyl-1,4-naphthoquinone[14,15].

Vitamin K_3 – a structural analogue of lawsone – is known to act as the cofactor for nitrate reductase[16,17]. Slight stimulation observed at lower concentrations in vitro (figure 1), though not very well understood, could possibly be due to lawsone functioning as a cofactor for nitrate reductase. Lawsone thus appears to be a very effective fungicide, for it kills the pathogen[4,10] on the one hand, and stimulates the nitrate reductase activity of the crop plants on the other[18]. A direct correlation between nitrate reductase activity and crop yield has recently been established[19].

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Giant collagen fibres in the gonopodium of the mosquitofish *Heterandria formosa* Agassiz, 1853 (Pisces, Poeciliidae)[1]

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Summary. The gonopodium of the mosquitofish *Heterandria formosa* was studied by transmission electron microscopy. In cross-section the gonopodium shows the following structure from the outside inwards: mutilayered epidermis, basal lamina and central supporting tissue, in which vessels and nerves are embedded. At the top of the gonopodium giant collagen fibres are found, which measure up to 150 µm in length and 6 µm in diameter. These fibres reinforce the gonopodium.

The gonopodium of the mosquitofish *Heterandria formosa* was studied recently in detail not only morphologically but also electron-microscopically[2,3]. The gonopodium of poeciliid fish represents a considerably modified analis, which is formed by the rays III–V.

In cross-section the gonopodium shows the following structure from the outside inwards: multilayered epithelium, basal lamina and central supporting tissues of varying structure (connective tissue and bony tissue consisting of osteoblasts, osteocytes and osteoclasts). Blood vessels and nerves are embedded in the supporting tissue.

The gonopodia were fixed and treated using methods which have already been published[4]. The specimens were decalcified with EDTA for 3–4 weeks[5]. The gonopodia