J. mar. biol. Ass. U.K. (1963) 43, 643–652
Printed in Great Britain

THE EFFECTS OF 3-AMINO-1,2,4-TRIAZOLE ON THE GROWTH OF SPORELINGS OF MARINE RED ALGAE

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(Plate I and Text-figs. 1–3)

The compound 3-amino-1,2,4-triazole (3AT) is now widely used as a herbicide and defoliant. There is considerable interest in the effects of this substance on plant metabolism, and numerous studies have been carried out with different crop plants. Rogers (1957) has shown that the chlorosis which follows application of 3AT to plants results from inhibition of chloroplast formation and Sund (1960) has suggested that this effect on chloroplasts may be due to the blocking of riboflavin synthesis; in addition he indicated some effect of 3AT on purine metabolism. Pyfrom, Appleman & Heim (1957) have shown that low concentrations of 3AT have no effect on the chorophyll content of mature leaves, but its influence on developing plastids causes a permanent reduction in the leaf's ability to manufacture chlorophyll. Wolf (1960) has also shown that the effects of 3AT on plastid pigments are secondary to its effects on the plastid itself.

In addition to inhibition of plastid formation, it is evident that other plant biochemical processes are markedly influenced by 3AT. Increased respiratory rates have been reported for cotton-leaf discs by Miller & Hall (1957), for bean and wheat leaves by Wort & Shrimpton (1958), and for grass seedlings (Russell, 1957). A marked lowering of the rate of photosynthesis has also been described (Wort & Shrimpton, 1958). Evidence for the effects of 3AT on enzyme activity has been recorded by Pyfrom *et al.* (1957). It would appear that application of 3AT alters the phosphorus economy of the treated plants. This has been demonstrated by Herbert & Linck (1958) and Wort & Loughman (1961). The effect on phosphate metabolism is confined to the processes involved in the incorporation of phosphate during nucleic acid synthesis.

Fewer investigations have been carried out on the effects of 3AT on algae. Frederick & Gentile (1959) have demonstrated its inhibitory effect on phosphorylase extracted from a blue-green algae, and more recently Wolf (1962) has shown that the growth inhibition of *Chlorella* brought about by 3AT can be reversed by addition of various purines. The pigmentation of *Chlorella* is much the same as that of higher plants, and there appears to be no infor-

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mation on the effects of 3AT on the growth of algae which possess distinctive accessory pigments.

The present work reports on the effects of 3AT on the growth of sporelings of five species of marine red algae. This forms part of a study of the effects of various biologically active compounds on sporeling growth, and an earlier paper has reported on the growth-stimulatory effects of some carcinogenic agents (Boney & Corner, 1962*b*). The inhibitory effect of 3AT on chloroplast formation would also appear particularly relevant to earlier studies on the possible function of accessory pigments in relation to the growth of red algal sporelings (Boney & Corner 1960, 1962*a*, 1963).

METHODS

Plant material and spore settlement. The five species used in the present work were (1) *Antithamnion plumula* (Ellis) Thur.; (2) *Plumaria elegans* (Bonnem.) Schm.; (3) *Callithamnion tetricum* Ag.; (4) *Nemalion multifidum* (Web. et Mohr.) J. Ag.; (5) *Brongniartella byssoides* (Good. et Woodw.) Schm. The plants were taken from a range of habitats. Thus, (1) was collected from a persistent summer growth in the Drake's Island Tank at the Plymouth Laboratory; similar material was the 'light-adapted' species used in some earlier experiments (Boney & Corner, 1963). (2) was collected from the sub-flora below *Ascophyllum* on Church Reef, Wembury; (3) was collected from landward crevices just above the region of the sublittoral fringe at Wembury, and (4) was collected from the exposed open rock in the same locality as (3), but from the region above the sublittoral fringe on the 'south peaks' of Church Reef (Colman 1933); (5) was dredged from the sea-bed in the vicinity of the Duke Rock buoy in Plymouth South (depth 10–12 m). Tetrasporangium-bearing plants were used in all instances with the exception of *Nemalion*, which produces only carpospores. Methods of spore settlement were carried out as described in earlier papers (Boney, Corner & Sparrow, 1959; Boney & Corner, 1959; Boney, 1960*a*).

Measurement of growth. Growth was measured in terms of cell production per sporeling crop. With *Antithamnion*, *Plumaria* and *Callithamnion* the same methods were used as in earlier studies (Boney, 1960*b*, 1962; Boney & Corner, 1960, 1962*a*). Because of the formation of pericentral cells in sporelings of *Brongniartella* growth was measured as described elsewhere (Boney & Corner, 1963). Measurement of cell production by *Nemalion* sporelings is possible because of the filamentous mode of early development (Pl. 1, *a–d*).

Culture medium. The culture medium used in the experiments consisted of autoclaved filtered sea water, enriched with 0·2 g $NaNO_3$ and 0·03 g $Na_2HPO_4 12H_2O$/l., and with Na_2 E.D.T.A. and trace elements as in Droops' S 20 medium (Droop, 1955). Solutions of 3AT were made directly in this medium. Spore settlements were carried out on rectangular pieces of glass

(2·5 × 1 cm) cut from glass slides, and culture vessels were 3 × 1 in. specimen tubes containing 20 ml. culture medium, and covered with plastic caps.

Optimum light intensity. Experiments were carried out in a constant temperature room (16° C) with lateral illumination from a vertically situated Mazda 'Daylight' fluorescent tube. Experiments with sporelings of *Plumaria* and *Callithamnion* were carried out at 10 ergs/sec/mm² in the wave band 380–720 mμ; sporelings of *Nemalion* and *Antithamnion* were grown at 17 ergs/sec/mm², and *Brongniartella* at 7 ergs/sec/mm². Whilst the optimum values for *Plumaria*, *Antithamnion* and *Brongniartella* have been determined by experiment (Boney & Corner, 1962*a*, 1963), optimum light intensity determinations were not made for *Callithamnion* and *Nemalion*.

Growth inhibition. This was measured as follows:

$$\left\{\frac{\text{Cells produced/day by control sporelings} - \text{Cells produced/day by treated sporelings}}{\text{Cells produced/day by control sporelings}}\right\} \times 100\,\%.$$

RESULTS

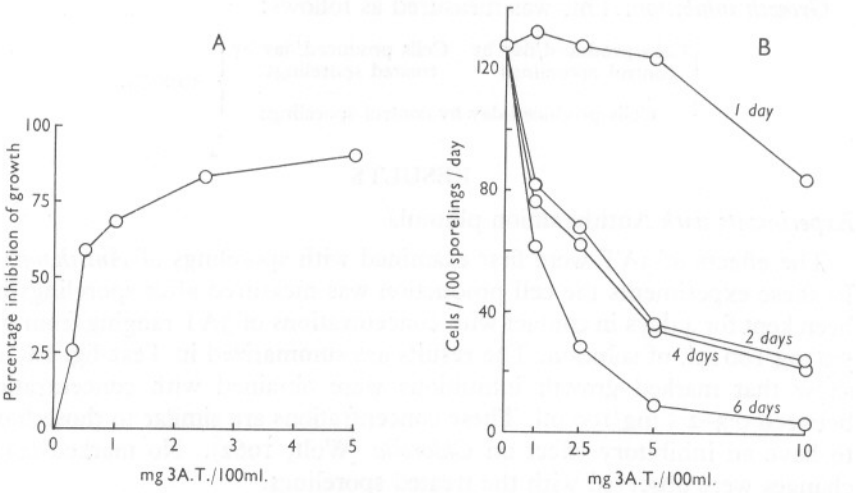
Experiments with Antithamnion plumula

The effects of 3AT were first examined with sporelings of *Antithamnion*. In these experiments the cell production was measured after sporelings had been kept for 4 days in contact with concentrations of 3AT ranging from 0·3–5·0 mg/100 ml. of solution. The results are summarized in Text-fig. 1A, and show that marked growth inhibitions were obtained with concentrations between 0·3–2·5 mg/100 ml. These concentrations are similar to those found to have an inhibitory effect on *Chlorella* (Wolf, 1962). No marked colour changes were observed with the treated sporelings.

In a second series of experiments the effects of immersing *Antithamnion* sporelings over different time intervals were measured using concentrations of 3AT ranging between 1–10 mg/100 ml. In these experiments the sporelings were immersed in the solutions of 3AT for 1, 2, 4 and 6 days. The overall period of experiment was 6 days, and samples immersed for the shorter periods were rinsed in filtered sea water and transferred to normal culture medium for the remainder of the experiment. The results of these experiments are summarized in Text-fig. 1B.

It is evident that immersion in 10 mg 3AT/100 ml. for 24 h caused a significant inhibition of cell production, but no effect was noticeable at lower concentrations. By contrast, immersion for 2 days in a concentration of 1 mg/100 ml. resulted in a similar growth inhibition (35%) to that obtained after immersion for 1 day in 10 mg/100 ml. 3AT; 2 days immersion in a concentration of 2·5 mg/100 ml. resulted in a 46% growth inhibition, and this was increased to 81% at 10 mg/100 ml. Growth inhibitions were a little higher at each concentration after 4 days immersion, and the results obtained after

6 days' immersion bear out those summarized in Text-fig. 1A, with almost a total inhibition of growth at 10 mg/100 ml. Interpreted in a different way, the results show that the mean rate of cell production of an untreated sporeling was about 1·3 cells/day. After 24 h immersion in 10 mg/100 ml. 3AT cell production fell to 0·84 cells/day for the remainder of the period of experiment. Similarly, 2 days' immersion at 10 mg/100 ml. resulted in the length of the filaments increasing by 0·25 cells/day, and sporelings immersed for 6 days in 1, 2·5, 5 and 10 mg/100 ml. produced 0·6, 0·28, 0·09 and 0·03 cells/day, respectively. Indications that 3AT induced some lasting effect on growth may also be deduced from these results. If it is assumed that growth was totally



Text-fig. 1A. Graph showing percentage inhibition of growth of *Antithamnion* sporelings with increasing concentrations of 3AT. B, Graph showing cell production per 100 *Antithamnion* sporelings per day measured after immersion in 3AT for different time intervals. (Each point the mean of four determinations).

inhibited during the period of immersion in the highest concentration used, then after 6 days a sporeling immersed for 1 day in 10 mg/100 ml. 3AT would show a mean cell production of (7·8–1·3) cells, or 6·5 cells. Measured at the end of 6 days, this would equal a growth rate of approximately 1 cell/day. By contrast, experimental results show that 0·84 cells were produced. Similarly, after 2 days' immersion the calculated value would be 0·85 cells/day, but the actual cell production was 0·25 cells/day. Added to this is the fact that a limited amount of growth is possible with sporelings immersed in this highest concentration over the whole of the experimental period. The measured inhibitions, therefore, are more than can be accounted for in terms solely of total cessation of cell production during the period of immersion in 3AT, and suggest instead that the results of contact with the compound will

still influence the rate of growth after sporelings have been transferred to normal culture medium.

Reversal of growth inhibition by addition of a purine

In experiments with *Chlorella*, Wolf (1962) observed that the growth inhibitory effects of 3AT could be reversed by the addition of various purines. In the present series of experiments this effect was investigated by adding adenine to media containing 3AT. The results are summarized in Table 1, and these show that the inhibitory effect of 3AT on the growth of *Antithamnion* sporelings is reversed by the addition of suitable concentrations of adenine.

TABLE 1. CELL PRODUCTION OF *ANTITHAMNION* SPORELINGS IN THE PRESENCE OF 3AT AND ADENINE

	Experimental conditions as described in text Cells/100 sporelings/day		
Culture medium	Culture medium + 1 mg adenine/100 ml.	Culture medium + 2 mg 3AT/100 ml.	Culture medium + 2 mg/100 ml. 3AT + 1 mg 100 ml. adenine
130 ± 2	128 ± 1	40 ± 1	122 ± 1

Effects of immersion in 3AT over longer periods

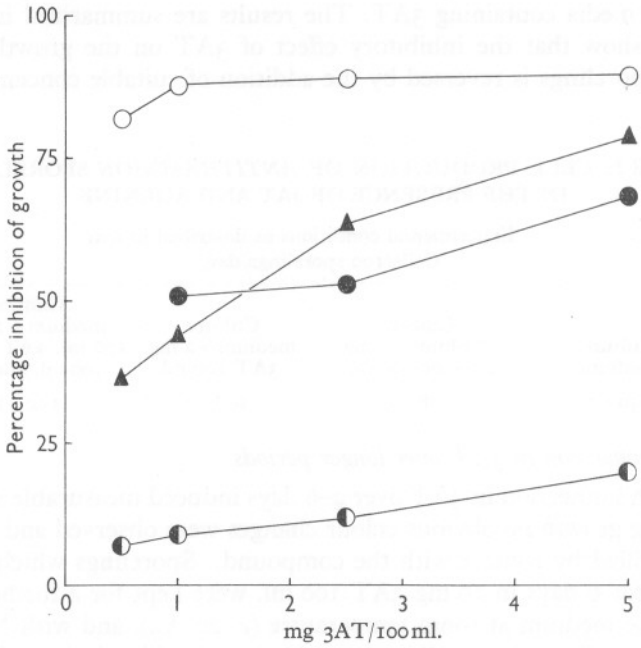
Although immersion in 3AT over 4–6 days induced measurable inhibitions of sporeling growth no obvious colour changes were observed and sporelings were not killed by contact with the compound. Sporelings which had been immersed for 6 days in 10 mg 3AT/100 ml. were kept for a further 12 days in this same medium at room temperature (*c.* 20° C.), and with N. window illumination. Control sporelings were kept under identical conditions. At the end of this period cell production per 100 sporelings was estimated. Sporelings immersed in 3AT produced a total of 1075 cells per 100 sporelings, whilst the control sporelings gave a cell total equal to 102,100 cells per 100 sporelings. Not only was this inhibitory effect still a very marked one, but in addition cells of the treated sporelings were pale green in colour, although showing no signs of death. Clearly protracted immersion in 3AT has a marked influence on the pigmentation of the plants.

Results with other species of marine red algae

The inhibitory effects of 3AT on the growth of sporelings of four other species of red alga were tested using similar conditions of experiment. Results are summarized in Text-fig. 2. These show that a marked inhibitory effect similar to that obtained with *Antithamnion* sporelings was observed with sporelings of *Plumaria elegans*, and that the cell production of sporelings of *Callithamnion* and *Nemalion* was also markedly inhibited. The least effect was

observed with sporelings of *Brongniartella byssoides*. Sporelings of this species showed only a 20% growth inhibition in concentrations of 5 mg/100 ml.

Of these four species, *Brongniartella* is the only one generally found in sublittoral habitats on the south-west coast. It is therefore of interest to note that the four intertidal species appear to be more influenced by contact with 3AT. Similarly the *Antithamnion* material used was that well-adapted to



Text-fig. 2. Percentage of growth shown by sporelings of various red algae with increasing concentrations of 3AT. —○—, *Plumaria elegans*; —●—, *Nemalion multifidum*; —▲—, *Callithamnion tetricum*; —◐—, *Brongniartella byssoides*.

almost normal daylight conditions (Boney & Corner, 1963), and the degree of inhibition recorded with this species was much the same as that for the intertidal representatives. This suggests, therefore, a different type of response on the part of sublittoral representatives from that shown by sporelings of algae which are subjected to some unfiltered daylight in their normal habitats.

DISCUSSION

Studies on the effects of 3AT on crop plants have indicated that a number of essential processes are influenced. These include chloroplast formation, respiration and photosynthesis rates, incorporation of phosphate during nucleic acid synthesis, and effects on enzyme activity. Whilst inhibition of chloroplast formation is a factor of considerable importance, the growth

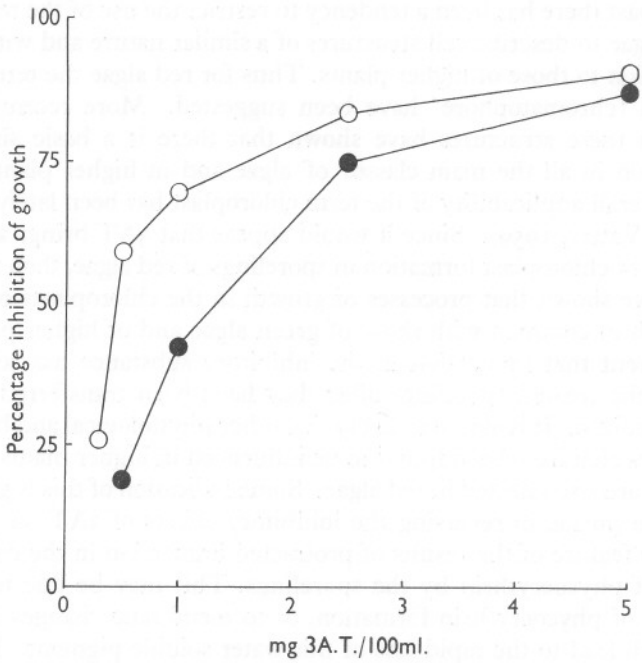
inhibitions obtained in the experiments with sporelings of red algae could be due also to 3AT influencing any of the other processes outlined above. However, the results of protracted immersion in solutions of the compound include a marked chlorosis of the sporeling cells, and this would imply that plastid formation is influenced in these representatives as observed with green algae and with higher plants.

In the past there has been a tendency to restrict the use of the term chloroplast in algae to describe cell structures of a similar nature and with a similar pigmentation to those of higher plants. Thus for red algae the terms 'rhodoplast' and 'chromatophore' have been suggested. More recently detailed studies on these structures have shown that there is a basic similarity of construction in all the main classes of algae and in higher plants, and the general overall applicability of the term chloroplast has been lately proposed, (Brody & Vatter, 1959). Since it would appear that 3AT brings about some inhibition of chloroplast formation in sporelings of red algae, then the present results have shown that processes of growth in the chloroplasts of red algae have much in common with those of green algae and of higher plants. It is also apparent that immersion in the inhibitory substance has some lasting effect on the treated sporelings after they have been transferred to normal culture medium. It is also very likely that other physiological and biochemical processes which have been found to be influenced in higher plants by contact with 3AT are also affected in red algae. Some indication of this is given by the action of a purine in reversing the inhibitory effects of 3AT on growth. A noticeable feature of the results of protracted immersion in the compound is the loss of phycoerythrin by the sporelings. This may be due to an actual inhibition of phycoerythrin formation, or to membrane changes induced by 3AT which lead to the rapid loss of this water soluble pigment. However, a severely limited amount of growth appears possible in the absence of this accessory pigment whilst sporelings remain in contact with 3AT.

The different response to contact with the inhibitory compound obtained with sublittoral and intertidal representatives is also of interest. Recent work, (Boney & Corner, 1960, 1962*a*, 1963) has suggested that the role of phycoerythrin in the early development of sporelings of intertidal red algae may be more concerned with protecting the young plants from the harmful effects of excessive green light, whereas in sublittoral representatives it would seem that the red pigment has the more accepted function of complementing the colour of the predominating wavelengths and so energizing chlorophyll *a* by resonance transfer of energy.

One way of testing whether sublittoral representatives do indeed show a different response to contact with 3AT would be to compare the effects of the compound on sporelings of an intertidal representative with sporelings of the same species, but from sublittoral habitats. This was done by comparing the effects of the compound on sporelings of *Antithamnion plumula*, using the

light-adapted material already described and plants of identical morphological form taken from depths of 10–12 m in Plymouth Sound. The results are shown in Text-fig. 3, and it may be seen that the effects of immersion in the lower concentrations of 3AT are far less marked with sporelings of the sublittoral representative than with the 'light-adapted' material.



Text-fig. 3. Percentage of growth shown by sporelings of *Antithamnion plumula* from different habitats. —○—, 'light adapated' material (see text); —●—, sublittoral material.

There is evidence for the fact that 'deep-water' red algae have a higher content of phycoerythrin than chlorophyll. Lebedev (1958) for example states that the sublittoral *Phyllophora nervosa* contains four to six times more phycoerythrin than chlorophyll. The results of experiments in the present work have shown that immersion in 3AT over a period of 6 days has a less marked effect on sporelings of sublittoral species than on intertidal representatives (placing the 'light-adapted' *Antithamnion* in the latter group). It may be that with these sublittoral algae inhibition of chloroplast formation (and of chlorophyll) has a less marked effect over the relatively short period of experiment because the initially higher phycoerythrin content of the spores and young sporelings leads to the red pigment being the main source of photosensitization without the intervention of chlorophyll *a*. Such an explanation of the function of phycoerythrin has been put forward by Haxo & Blinks

(1950). However, with intertidal species where there is a greater reliance on direct sensitization of chlorophyll *a*, inhibition of chloroplast growth and chlorophyll formation has a more immediate effect on the growth of the sporelings.

The algae form a heterogeneous assemblage of plants, and in recent years much information has emerged concerning the various physiological and biochemical processes leading to the formation of the distinctive cell wall constituents and reserve products characteristic of the main algal classes. Information which tends to underline basic similarities in certain growth processes are therefore of some value. Furthermore, it may be stressed that conservation problems associated with the widespread use of new and potent herbicides should not be interpreted solely in terms of higher plants, but must also take into account possible side effects on natural cycles in which aquatic algae play a fundamental part.

SUMMARY

The cell production of sporelings of five species of marine red algae is inhibited by 3-amino-1,2,4-triazole (3AT).

Results show that short-term immersions in culture medium containing 3AT have a lasting effect on the growth of sporelings.

This growth inhibitory effect is reversed by addition of adenine.

Protracted contact with 3AT results in some chlorosis of the sporelings.

The growth inhibitory effects of 3AT are more marked with sporelings of intertidal red algae than with sublittoral species.

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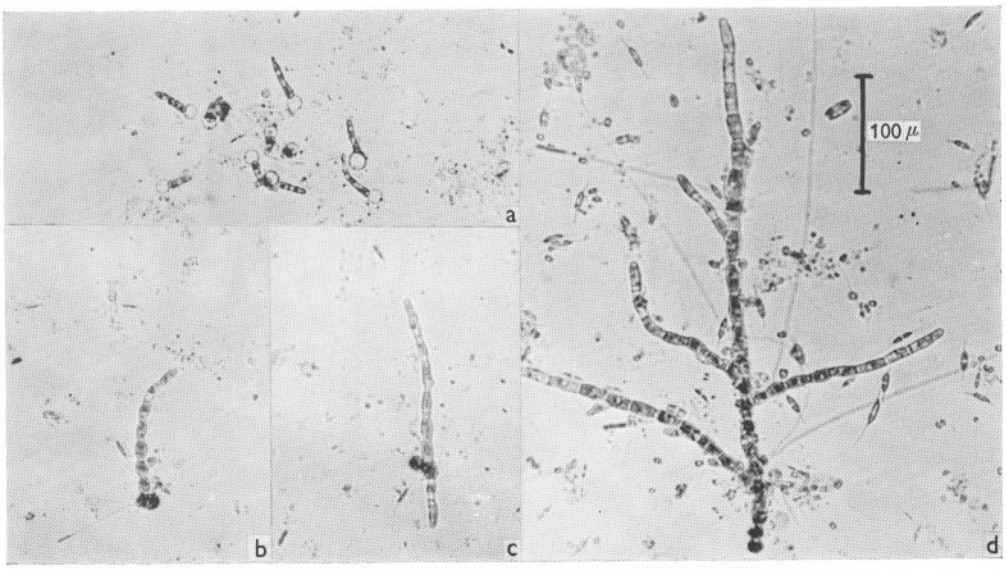
EXPLANATION OF PLATE 1

Development stages of *Nemalion* sporelings: *a*, 3 days after settlement; *b*, after 5 days growth; *c*, after 7 days growth; *d*, after 15 days growth.



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