

FURTHER OBSERVATIONS ON THE FUNGICIDAL ACTION OF BORDEAUX MIXTURES.

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IN earlier papers (this *Journal*, vol. iv. pp. 69 and 76) we have detailed the experimental evidence which led us to conclude (1) that the view as to the fungicidal action of Bordeaux mixtures favoured by Pickering, viz. the liberation of copper sulphate by *atmospheric* carbon dioxide, is untenable; and (2) that contact between the fungus and the copper compound present in the mixture will account largely for its efficiency owing to a solvent action on the part of the organism under certain conditions. Pickering (this *Journal*, v. p. 273) has criticised our general conclusions and the deductions which we have drawn from certain of our experiments; and therefore before describing our further work on the subject, a brief reference to some of the points which he has raised is desirable.

Pickering now apparently accepts the view that the fungicidal action of Bordeaux mixtures cannot be attributed to the formation of copper sulphate, as he formerly maintained; for he attempts to demonstrate, by analogy with the results of experiments with metallic iron, that it is the slight solubility of the copper compound in water which is responsible for the action. Objection is raised to our experiments with spores on the ground that "the excretion of a solvent substance from them could only be established by examining their action on a copper compound which is really insoluble in water, and this Barker and Gimmingham have not done, all their experiments having been performed with 10 CuO, SO_3 which is not insoluble." He has evidently overlooked the statement (p. 86, *loc. cit.*) that ordinary Bordeaux (containing the

insoluble compound $10 \text{ CuO}, \text{SO}_3, 3 \text{ CaO}$) was also used in certain experiments. His comparison of the results with spores and those which can be obtained with metallic iron, on the assumed similarity of which he bases the whole argument of the remainder of his paper, therefore falls to the ground, since according to his own statement the compound $10 \text{ CuO}, \text{SO}_3, 3 \text{ CaO}$ does not itself act on iron. We have demonstrated that contact between it and the fungus can result in the death of the latter, even in the presence of a great excess of lime (as in ordinary Bordeaux mixture) under circumstances precluding the possibility that all the lime had been carbonated. This, on Pickering's own showing, therefore, can only be explained by the assumption of a solvent action. Further, the results of his experiments on the action of $10 \text{ CuO}, \text{SO}_3$ on iron rods in the presence of atmospheres containing different amounts of carbon dioxide show that the presence of that gas in quantities likely to occur under practical conditions *retards* rather than favours the solution and removal of the copper. (See Table, p. 278, *loc. cit.*)

He admits that there should be a differentiation between the action of carbon dioxide and of ordinary air on the Bordeaux precipitates, but suggests that "it is questionable how far such a differentiation is of importance from the point of view of practical spraying, for any fluid sprayed on to the leaves of trees will soon find itself bathed in an atmosphere of carbon dioxide evolved from the leaves themselves." This assumption of the presence of an atmosphere of carbon dioxide in the neighbourhood of leaves can surely not be accepted without proof, in view of the many factors which must tend to a rapid dispersal of the gas out-of-doors.

The following experiment bears on this point. All the leaves of a large strawberry plant in a pot were completely covered with a paste of the basic sulphate $10 \text{ CuO}, \text{SO}_3$. The plant was then placed in a bowl of water under a bell-jar in order to keep the atmosphere saturated. Under these conditions the action of any CO_2 respired by the leaves would presumably be at a maximum.

After three days, as much as possible of the transpired and condensed water on the leaves was shaken off into a small beaker. 2 or 3 c.c. of liquid were collected in this way. No dissolved copper could be detected in this liquid by the ferro-cyanide test. The leaves were then washed with distilled water and the wash water tested for copper, again with a negative result.

The experiment was repeated keeping the plant in darkness with the same result.

222 *Fungicidal Action of Bordeaux Mixtures*

Again, stress is laid on the fact that a drop of Bordeaux mixture placed on a piece of paper soaked in potassium ferrocyanide solution gives the red colour of copper ferrocyanide as it dries up; and that further the red colour does not develop if the air in contact is deprived of its CO_2 . We have specially referred to the action of atmospheric CO_2 (p. 74, *loc. cit.*). The ferrocyanide test is only given when the solid particles of the Bordeaux precipitate are in close contact with the paper, for no visible colour appears when ferrocyanide paper is dipped into the clear liquid above a Bordeaux precipitate, even if a current of air (containing the normal amount of CO_2) has been passed through the mixture previously for 5—6 hours. The results of such experiments do not, however, affect the question of the fungicidal action, because, as we have recorded (pp. 87 and 88 *loc. cit.*), spores will germinate and grow in the liquid in presence of the copper precipitate so long as they are not in actual contact with or sufficiently close to the actual particles¹.

Our experiments have failed, according to Pickering, to establish "the excretion of solvent matter from fungus spores." Our point was not so much that fungus spores in general possessed solvent properties, as that, whilst all living cells were capable of producing solvent material, the actual factor which determined the extent of the solvent action was the degree of permeability of the external cell wall concerned. Indeed, Pickering's description of the experiments with dried film cover-slip preparations to which we attached considerable importance (p. 88, *loc. cit.*) is quite inaccurate; and the results which we then obtained have been abundantly confirmed by the work shortly to be described.

Elsewhere in his paper, Pickering suggests that our results with ordinary Bordeaux in an atmosphere free from carbon dioxide were due to the fungicidal action of the excess of lime present, basing his statement on some results obtained by Foreman (this *Journal*, III. p. 400). Reference to the latter paper shows however that to claim "a conspicuous fungicidal action" on the part of the lime from these results is hardly justifiable.

Finally, the contention "that if fungicidal action depended on a solvent material exuded from the fungus, all copper compounds, or at any rate all the basic sulphates and carbonates, would be equally efficacious for a given weight of copper present, a proposition which is

¹ In view of this, we have throughout recorded as *nil*, any amount of copper less than that which will give a visible colour with ferrocyanide, although fully aware that in many cases by concentration of the liquid, copper might have been detected in solution.

contrary to all experience," assumes that the compounds are all equally susceptible to a given solvent (which is certainly not true as regards water) and at the same time ignores many other factors which are involved. For instance, there are manifest and well known differences in the solubilities and physical condition of the basic sulphates and carbonates of copper, to some of which Pickering himself has frequently drawn attention. These are amply sufficient to account for the observed differences in efficiency; and, indeed, from the practical point of view, the outcome of our work is to direct more attention to the importance of the physical properties of Bordeaux sprays than to their chemical nature.

We do not then consider that our main contention as to the cause of the fungicidal action of Bordeaux mixture has in any way been weakened by Pickering's criticisms. Indeed, in view of our further work we maintain even more strongly that the part played by the living cell in relation to the insoluble copper compound is the important point.

Experiments with Fungus Spores.

§ 1. In the first place the experiments with fungus spores described in our previous paper were repeated and amplified. The general results were precisely the same as those already recorded. The conidia of *Nectria ditissima*, whether the surrounding air be freed from CO₂ or not, are killed by Bordeaux mixtures when there is actual contact between the fungus spore or germ-tube and a particle of the copper compound; but, on the same coverslip and in the same film of liquid where there is no such contact, even though the conidia may be not more than their own length away from a copper particle, the organism is unharmed. The toxic action of the copper where it occurs is rapid, death of the cell ensuing in less than 12 hours. These results have been obtained with copper compounds of different degrees of insolubility including several basic sulphates and carbonates.

It is difficult to explain the results satisfactorily unless a solvent action on the part of the living cell is assumed. Pickering's explanation that death is due to absorption of minute traces of copper in solution in the continuous film of liquid covering the preparation absolutely fails to explain such cases. Still less can it explain the fact (also referred to previously) that the septate *Nectria* conidia may show death of one or more of the constituent cells, while the remainder may live and produce germ-tubes. Such localised effects indicate most

clearly the existence of a definite solvent action at the points concerned.

In some instances, it is true, groups of conidia, though not in actual contact with any particles of the copper compound, failed to make much growth and eventually died. It is probable that although a single cell may fail to secrete sufficient of the solvent to act at a distance from the copper, yet the combined efforts of a group of cells may well be successful.

Further experiments with thick-walled spores have confirmed the results with uredospores of *Puccinia* which were recorded in our earlier paper.

We have therefore as a result of these experiments definite evidence indicating a solvent action on the part of the living cell, the conditions determining the toxic effect being the quantity of the solvent substances passing out of the cell, the distance between the cell and the nearest particle of the copper compound and the amount of the latter within the sphere of action. In addition, the time factor must be taken into account. If the rate of growth of the cell and of the formation of new cells exceed the rate of production and absorption of the toxic dose of dissolved copper, death of the whole organism will not occur, although individual cells may succumb.

Experiments with other types of cells.

§ 2. In order to gain more light upon the general action of copper fungicides and, if possible, to obtain further evidence in support of our original conclusions, a number of experiments have been made upon the action of copper compounds on plant cells other than those of fungi. Before discussing these, we should like here to acknowledge the assistance given us by Mr J. W. Eves, who set up many of the experiments.

In the first place it was found that the root-hairs on the primary roots of seedlings could be utilised conveniently for such experiments; and in many respects they may be considered as types of cells comparable with the germ-tubes of fungus spores or the cells of actively growing hyphae. After a trial with several kinds of seedlings it was decided to work chiefly with those of the ordinary Broad Bean, since these proved to be very suitable; and the following observations refer to experiments with Broad Bean seedlings, unless it is otherwise stated.

When the radicle has grown to the length of an inch or so, root-hairs in all stages of development are to be found. The roots of some seedlings at this stage were placed in contact with Bordeaux mixture, great care being taken to avoid damage to the delicate root-hairs. The seedlings were then left overnight, and on the following morning it was possible to see quite clearly under a low power that in very many cases the protoplasmic contents of the root-hairs were dead. Often, not only had death occurred but the contents of the cells had become strikingly stained with a purplish blue colour, evidently due to the formation of some compound with the absorbed copper.

All the root-hairs were, however, not killed and some, as far as microscopic examination showed, not even injured. Assuming the existence of direct solvent action on the part of the cell, this is precisely the result to be anticipated, since the methods adopted would not cause particles of the basic copper sulphate to adhere to all of the root-hairs nor equal amounts to each. Hence the variability in the behaviour. If the injurious action was due to the production of soluble copper by agencies other than the living cell itself, the whole root-hair system should have been more or less equally affected instead of showing the extreme variations recorded.

§ 3. Other immersion experiments serve to complete the chain of evidence for the direct cell action hypothesis.

If the Bordeaux mixture precipitate is allowed to settle and the roots placed carefully in the supernatant clear liquid, on the day following the root-hairs are all apparently uninjured. (After several days, abnormal changes and death ensue; but as will be seen later, this is readily explained under the direct cell action hypothesis.) The case is even more striking if two seedlings are placed in the same vessel so that one has its root dipping into the precipitate at the bottom whilst the root of the other remains in the clear liquid above. After 24 hours many of the root-hairs of the former are killed, whilst those of the latter are all uninjured. These results have been confirmed with pea seedlings.

It is evident therefore that it is the direct contact between the thin-walled root-hair and the basic copper sulphate which causes the death of the cell within a few hours. In other words, there must be at work a reciprocal action between the cell and the insoluble copper compound when the two are in contact, which becomes inappreciable or is altogether absent when they are not in actual contact. The results are exactly analogous with those obtained with fungus cells. It is

therefore fair to conclude that living plant cells with walls of the type which permits ready passage of diffusible substances, are capable of exerting a direct solvent action on the insoluble copper compounds of Bordeaux mixture, provided the particles are sufficiently near to the cell.

§ 4. In experiments such as those just described, where the young root is brought into contact with the basic copper sulphate, not only do the root-hairs suffer in the manner indicated, but any part of the root in contact with the copper compound also becomes blackened. This is the case both with bean and pea seedlings. If the root tip rests in a pasty mass of the basic sulphate no further apical growth occurs. The portion of the primary root above the paste elongates and new secondary roots arise laterally from that region. These develop naturally until in their normal downward direction they strike the paste, when they suffer the fate of the primary root. The same kind of thing is repeated until in the end the whole root system is represented by a short knotted and blackened stump. Copper can be detected in the ash of the aerial parts of such plants.

§ 5. Again, if the paste of basic copper sulphate is applied to the young root in zones or bands, leaving intermediate portions untouched, the result is a blackening of the superficial cells on the treated areas within 24 hours, whilst the untreated areas remained unaffected.

§ 6. If bean seedlings are grown in water culture, using Bordeaux mixture, occasionally stirred, as the culture fluid, blackening of the roots ensues as in other cases of direct contact with the basic copper sulphate. On the other hand, if the Bordeaux mixture precipitate is allowed to settle before the introduction of the seedlings, the roots remain unaffected and continue in active growth for days in the clear liquid. When, however, such cultures are kept for some time, changes begin to appear. As the roots approach the layer of copper compound at the bottom of the vessel, the downward growth is checked and altered to a horizontal or even upward direction, away from the region of the copper. Under these circumstances the tips of the roots rarely actually penetrate into the deposit; and eventually after two or three weeks the roots in the clear portion of the liquid begin to show signs of blackening. Active growth ceases and finally the whole root system slowly acquires a knotted appearance, due to the attempted formation of secondary roots, the growth of which is almost at once arrested. Evidently, under the conditions of this experiment, no diffusion being possible beyond the limits of the vessel in which the plants were growing, there is an accumulation of the excreted products of the cells which, sufficient time

being given, finally reaches a concentration sufficient to act on the copper compound, even though it is situated at a slight distance.

§ 7. Many of these experiments were repeated in still more conclusive fashion, by placing the Bordeaux mixture inside a diffusion tube which was then partly immersed in a larger vessel containing the roots of the seedlings in the filtrate from Bordeaux mixture. Here there could be no possibility of direct contact between the roots and the particles of the copper compound. Control cultures without Bordeaux mixture were included. The results were the same as those of the experiment just described; the roots remained healthy and in active growth for some days, as in the controls, but eventually became discoloured and deformed.

§ 8. The same points were exceedingly well illustrated by experiments with mustard seedlings. Some strips of moist flannel were covered either completely or in isolated bands with the basic copper sulphate in the form of a paste. Mustard seeds were then sown fairly thickly over the whole surface and the flannel suspended with the ends dipping into water or Bordeaux mixture. The result was an excellent crop of seedling plants on the untreated areas which contrasted in a most marked manner with the ungerminated seeds and the few weak seedlings on the treated areas immediately adjoining. On the treated area, those seeds completely or almost completely immersed in the paste were killed before or immediately after germination. If however the micropyle of the seed was so situated that the radicle grew away from the paste, the total growth was sometimes sufficient to enable the seedling to withstand the shock when the radicle did eventually come into contact with the paste causing further apical growth to cease. Such cases were however rare, the great majority of the seeds getting scarcely beyond germination. The seedlings on the untreated area grew right up to the edge of the treated area; and equally good growth occurred if seeds were sown on the opposite side of a treated area, the seedlings then being removed from the paste only by the slight thickness of the flannel. Further, the seedlings grow freely and vigorously if distributed over the outside of a diffusion tube containing the basic copper sulphate paste.

§ 9. In all cases the injurious action of the paste on living cells is entirely local and cannot be explained as a result of the production of soluble copper by atmospheric agencies. The action is not limited to the basic copper sulphate formed in the "no-excess-lime" Bordeaux mixture ($10 \text{ CuO}, \text{SO}_3$), though this compound has been used in the

majority of the experiments. The same results are obtained with the compound present in ordinary Bordeaux mixture (10 CuO, SO₃, 3 CaO) and with copper carbonate. With the last mentioned substance, however, the solvent action of the cells is apparently less vigorous.

The Influence of the Nature of the Cell Wall.

All experiments dealt with up to this point have been concerned with the action of cells with walls more or less readily permeable, and serve to prove the correctness of the direct cell action hypothesis in the case of this type of cell. Our further work directs attention to the nature of the cell wall as a most important factor in the whole question of the relation of Bordeaux mixture to plant life.

§ 10. The effect of covering the seed coats of beans and of peas with a paste of the basic copper sulphate shows the behaviour with permeable cell walls of a somewhat different kind from those already discussed. In the case of the bean, discolouration of the outer seed coat occurs (confined to the areas in actual contact with the paste), but, under suitable conditions, germination takes place in normal fashion and so long as the radicle does not come into contact with the paste the young plant grows like that from an untreated seed. The blackening and discolouration is entirely restricted to the seed-coat and does not penetrate through it to the cotyledons. On the other hand, the seed-coat of the pea remains almost colourless but permits ready passage of copper to the cotyledons which acquire a blue-green colour. Germination is very much weakened or, if the time of contact with the paste is at all prolonged, entirely prevented. The thick fleshy coat of the bean retains all or practically all of the absorbed copper and the embryo itself escapes any toxic effect, whilst the thin semi-transparent coat of the pea readily transmits the absorbed copper to the cotyledons. If the seed-coat of the bean is removed before treatment, results like those with the pea are obtained.

§ 11. The action of cell walls of an entirely different type has been studied by an investigation of the interaction between the Bordeaux mixture and the leaf cells to which it is applied in practical spraying. The following account of experiments with apple and potato and other foliage is concerned with the behaviour of cells with impermeable or comparatively impermeable walls, such as the cuticularized outer walls of the cells of the leaf epidermis, when they are brought into contact with various copper compounds.

In this work with foliage it became apparent at a very early stage that trustworthy results would only be obtained when the portion of the leaf selected for the test possessed a cuticular surface absolutely free from injury of any kind. Emphasis must be laid upon this point, since the risk of abnormal results is very great if any part of the cuticle coming within the area of application of the fungicide has suffered damage. Great care is necessary in the selection of a suitable surface for the test on account of the rarity of occurrence of absolutely undamaged leaves on plants grown under ordinary outdoor or greenhouse conditions. In a very large percentage of cases the injury is so slight or so minute as to be invisible to the naked eye: but, although it may be only microscopic, it frequently suffices to vitiate the results. For this reason much of the work done with ordinary exposed foliage has been checked by corresponding experiments carried out with uninjured leaves obtained by growing the whole plant or individual branches enclosed in muslin screens from the time of the bursting of the buds, thus protecting the foliage from the possibility of damage by bruising or by insect attack. The following observations are confined to those cases in which we have satisfied ourselves that leaf injury has played no part.

§ 12. In the first place, in order to obtain evidence with regard to the extent to which the cuticularized walls of epidermal cells are impermeable, a number of healthy summer leaves of various kinds of apples (as far as possible free from visible injury) were immersed in copper sulphate solution (1, 5 and 10 per cent.) for periods varying from 5 minutes to 24 hours. It was remarkable how very little general injury to the surface of the leaves resulted. The damage was invariably restricted to separate areas starting from centres irregularly distributed over the surface of the leaf and could be increased by prolonging the immersion. The epidermis of the leaf as a whole is certainly not appreciably permeable: there is no general and simultaneous injury to the whole leaf surface. The appearance indicated that where damage resulted it was due to the penetration of copper to the inner thin-walled mesophyll cells of the leaf at certain isolated points. Under the microscope it was almost always possible to trace an original small injury as the centre of each area of damaged cells. Experience has shown that an absolutely uninjured apple leaf is rarely if ever to be found on a tree growing in the open, at all events after the early summer.

§ 13. Working at our suggestion, Mr S. P. Wiltshire, B.Sc., eventually got over the difficulty of finding an uninjured cuticular

surface to test by isolating small areas of leaves proved to be free from injury by microscopical examination. This was done by attaching glass ring cells of the type used for drop cultures to the surface of the leaf with vaseline and filling them with the liquid to be tested, thus confining the action to the area enclosed by the cell. By this method the uninjured epidermis of the apple leaf was shown to have a very remarkable power of resistance to the penetration even of corrosive liquids. Exposure to the action of 20 per cent. copper sulphate solution or of Perenyi fluid (3 parts alcohol, 4 parts 10 per cent. nitric acid, 3 parts 0.5 per cent. chromic acid) for several days caused no detectable injury to the upper surface of the leaves; on the lower surface a slight yellowing was observed which proved to be due not to any effect on the cuticle proper but to a discolouration of the walls of the hairs with which the lower epidermis is covered.

§ 14. Corresponding experiments to those with solutions of copper sulphate have been made with Bordeaux mixtures and with a paste of the basic sulphate $10 \text{ CuO}, \text{SO}_3$. As was to be expected, there was little or no effect upon the general surface of the leaf. Only at spots where an injury (natural or made purposely) allowed contact between the copper compound and the inner thin-walled cells was there any noticeable discolouration of tissue or "scorching". On really uninjured leaves the hairs on the under surface were the only cells which showed the least trace of any absorption of copper; and this applies both to leaves treated with the basic sulphate paste and to those immersed in copper sulphate solutions. Several varieties of apples were tested in this way with no significant differences in the results. It should also be recorded here that Mr Wiltshire after a long series of examinations and measurements could find no sort of correlation between the thickness of the cuticle of the leaves of different varieties of apples and pears and their known susceptibility to scorching by Bordeaux mixture.

§ 15. One further point is of much interest in this connection. The observations described above no longer hold good when the experiments are repeated with autumnal foliage. After September distinct signs of an injurious effect of the copper treatment become apparent, both when Bordeaux mixtures or copper sulphate are used; and this effect is the more pronounced the later in the season the tests are made. General discolouration and scorching of the leaf surfaces, both

¹ Further work on the influence of injuries upon the "scorching" of foliage by Bordeaux mixtures and on the absorption of copper by plants is described in a paper shortly to be published in the *Annals of Economic Biology*.

upper and lower, quickly appear: and these are of quite a different character to those which occur locally at times during the summer experiments. Premature defoliation also follows, and the cuticularised epidermal cells of the leaves are found to be generally stained a blue green colour. The general effect is so uniform that it would seem to indicate some change in the nature of the cuticularized wall which renders it permeable and capable of a solvent action upon the copper compound. At the same time, since in the late autumn many leaves are likely to be considerably damaged, it is just possible that injury to the cuticle is sufficiently widespread to account for the results.

When the same experiment is repeated, using copper sulphate solution instead of Bordeaux mixture, a considerable amount of copper is absorbed and finds its way through the leaves and into the stem discolouring and killing all the cells through which it passes. Copper was easily detected by the ferrocyanide test in the ash of parts of the discoloured stem and of leaves remote from the parts actually treated with the copper sulphate solution¹.

§ 16. These experiments serve to show that the cuticularized walls of the epidermal cells of apple leaves in the summer stage are examples of typical impermeable walls. Their behaviour in relation to Bordeaux mixture as compared with that of unchanged cellulose walls, such as those of the root-hairs and roots of seedlings, may be considered analogous to that of certain thick walled resistant fungus spores in comparison with that of thin-walled germ tubes and actively growing hyphae. It is true that the impermeability of the wall of a fungus spore cannot be demonstrated so conclusively as that of a cuticularized epidermal wall; but it will probably not be disputed that the resistant spore wall is the fungus equivalent for a cuticle and therefore more or less impermeable so long as the spore is in a resting condition.

General Conclusions.

From the results here recorded it may be concluded that:

1. Living cells with readily permeable walls of the unchanged cellulose type or its equivalent are able to produce and absorb soluble copper from insoluble compounds such as the basic sulphates. (§§ 1—8.)
2. The area over which a single cell can exert the solvent action is

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limited by the size of the cell or, perhaps more accurately, by the quantity of the solvent diffusing from it. Groups of cells acting in conjunction may cause appreciable action over a wider area than an isolated cell acting singly. (§§ 6, 7.)

3. The fate of the organism depends upon the relation between the amount of soluble copper produced and absorbed and the rate of growth of the organism. This is a significant point in connection with practical spraying, since it explains why there may be at times little check to the growth of a parasitic fungus after spraying, especially when the parasite has once gained a footing on the host-plant. (§ 8.)

4. Cells with walls of an impermeable character possess no such power of solvent action upon insoluble copper compounds. In the case of apple leaves only when there is injury to the cuticle sufficiently recent for no occlusion to have taken place, or when there is some radical alteration in its nature, is soluble copper produced, with attendant "scorching" or local injury of the exposed thin-walled cells of the subjacent tissues. (§§ 12—14.)

5. Under changed conditions, cells with normally impermeable walls may become permeable and capable of action upon insoluble copper compounds. The difference in behaviour of summer and autumn apple foliage would seem to be best explained in this way; and the change in the nature of the cell wall may perhaps reasonably be attributed to incipient death of the cells preparatory to leaf fall. This explanation fits in well with the fact that the hairs on the under surface of apple leaves (which are decadent cells) are affected by contact with the copper compound even in early summer, when the epidermal cells (being full of life and vigour) remain unattacked. (§ 15.)

It is evident therefore that the nature of the cell wall is the determinative factor in the matter of direct action of the cell upon the Bordeaux compounds. Comparison of the conclusions here stated with those derived from our original work with cells of fungi and its repetition and extension described earlier in the present paper shows that two distinct lines of work have led to identical results. The study of seedlings and foliage in relation to Bordeaux mixture has thus furnished the strongest support for our views as to the fungicidal action of that spray fluid.