SEED DISINFECTION FOR PURE CULTURE WORK: THE USE OF HYPOCHLORITES

B. M. DUGGAR

Physiologist to the Missouri Botanical Garden, in Charge of Graduate Laboratory
Professor of Plant Physiology in the Henry Shaw School of Botany of Washington University

AND ANNE W. DAVIS

Formerly Research Assistant to the Missouri Botanical Garden

INTRODUCTORY

For a long time it has been clear that much light may be thrown on many fundamental problems in seed plant nutrition as well as in physiological pathology through the use of pure cultures of certain seed plants. In those cases where the seed are produced in pods, solid fruits, or within other thoroughly protective coverings, it is, as a rule, a relatively simple matter to secure seed in season entirely free from contamination. It is only necessary to employ the usual bacteriological precautions, opening the maturing pods or fruits with care and removing the seed to sterile containers, in which they may be kept until required. Beans, peas, radishes, tobacco, tomatoes, and various cucurbits or melons are among those plants easily handled in this way. The difficulty, however, even with these seed, lies in anticipating what may be needed out of season. With the majority of seed, moreover, it would not be practicable to use the isolation method either because of structural difficulties or of inaccessibility of fruiting plants.

The experiments of Wilson[1] on the use of commercial chloride of lime (in part calcium hypochlorite) have been the first definite application of the value of this well-known disinfecting agent to plant physiological study with seed plants. In the disinfection and antiseptic treatment of wounds, extensive studies have been made during this war with the use of hypochlorous acid, the hypochlorites, and related compounds, as a result especially of the investigations of Dakin[2]

1 Wilson, J. K. Calcium hypochlorite as a seed sterilizer. Am. Jour. Bot. 2 : 420–427. 1915.

2 Dakin, H. D., and E. K. Dunham. A handbook on antiseptics. 126 pp. New York, 1917.

and his associates. In general, this work has emphasized the value of a series of compounds containing "active chlorine," by which term Dakin infers a connotation of "the ability of any particular substance to part with chlorine, free or combined, in such a way that it can effect the chlorination of bacterial and other proteins." Through the NH groups of their constituent amino-acids the proteins are subject to attack by such chlorinated agents, whereby in the first step the Cl is substituted for the H-atom in the group mentioned with the formation of chloramines.

Materials and Methods

In the preliminary work here reported we have not departed from the readily obtainable commercial products, namely, (1) commercial chloride of lime, or "bleaching powder," (2) "chlorinated potassa" (a liquid product recognized by certain St. Louis manufacturers as "eau de Javel"), (3) solid sodium hypochlorite, and (4) Dakin's soluble chloramine T. These we have compared with a few standard disinfectants of other groups. The commercial products vary somewhat in composition, but these differences, in our experience, are not so great as to interfere with this type of practical work. A careful study of standardized preparations is, however, planned. Chloramine T, or chlorazene, is the abbreviated or trade name for sodium-toluene-para-sulphochloramide. No experimental work was done with the other Dakin products of this class.

The chlorinated lime, designated 10 per cent, was prepared in the following way: Ten grams of a standard commercial product were stirred into 100 cc. distilled water. After standing 10 minutes the supernatant liquid was filtered and the filtrate employed. Other concentrations were prepared in an analogous manner. The commercial Javel water was used as if it were a pure substance, 10 and 20 per cent solutions referring respectively to the use of 10 and 20 cc. of the commercial product with enough water in each case to make 100 cc. All necessary precautions have been taken to prevent accidental contamination. The technique of handling treated seed

has been invariably carried out in a transfer room repeatedly steamed to insure the precipitation and fixation of dust particles. In some cases the seed were preliminarily immersed in running water for from 4 to 16 hours, in other cases dry seed were treated directly.

In the earlier work the selected seed were placed in small cheese-cloth bags, and these immersed in covered vessels containing the disinfecting solutions employed. After the interval of treatment the bags were transferred to jars of sterile distilled water for from 15 minutes to 1 hour, and when it seemed desirable a second quick rinsing was given. The contents of each bag was then carefully dumped into a sterile Petri dish. In later work the washed or soaked seed were carefully placed in sterile Erlenmeyer flasks, and the disinfectant then poured in, a separate flask being used for each lot of seed to be treated for any interval of time as well as by each concentration of disinfecting solution. After treatment the seed were twice shaken with sterile water, the second wash water remaining not less than 15 minutes. The seed were then transferred to a Petri dish by means of a metal spoon. All implements employed were sterilized by dipping in alcohol and then promptly burning this off.

The purity and germination of the seed were then followed after their transfer to large Petri dishes containing standard potato decoction agar. Care was taken to insure intimate contact of the seed with the medium. From 15 to 50 seed, depending upon size, were usually arranged in each dish, and each test duplicated.

Inasmuch as the essential thing in such work is to obtain a high percentage of germinating seed free from contamination and readily transferable to other cultures, the above methods have seemed entirely adequate, and complicated apparatus, such as that devised by de Zeeuw[1], is not merely unnecessary, but it is in general impracticable.

In the true sense of the word "control" experiments are not possible in this work, and no attempt is made to include ex-

1 Zeeuw, R. de. The comparative viability of seeds, fungi and bacteria when subjected to various chemical agents. Centralbl. f. Bakt. II. 31 : 4–23. *1 f.* 1911.

periments thus designated. All seed not disinfected will exhibit contamination on agar. On the other hand, elaborate experiments might have been made to determine the effect of the disinfectant upon the seed, and in a certain sense these would serve as controls. Our object, however, has been simply to use seed which under the usual conditions of the germinator exhibit a high percentage of germination, so we have merely assured ourselves of the capacity of the seed employed to germinate satisfactorily. It is admitted that germination in a Petri dish on agar is not comparable to germination in a germinator.

Experimental Data and Discussion

At the outset it should be definitely acknowledged that as a general principle the practically perfect disinfection of seed by chemical agents is only possible when the contaminating organisms of the seed are superficial, or largely superficial. This conclusion is drawn from a variety of observations and experiments, the general result of which is too obvious to require elaborate data and discussion but merits mention in respect to some pronounced instances. Experiments have been made with seed suspected of more than superficial contamination in the case of corn, sunflower, squash, tomato, and *Melilotus*. In the first case, corn of the 1917 crop was obtained in which a discoloration of the micropylar end of the seed was characteristic. The normal maturity of this crop in the Middle West was more or less affected by early frost, and in some sections subsequent wet weather induced visible mouldiness. The seed used exhibited no macroscopically visible infection of the cob, but such infection was inferred. After treatment with various disinfecting agents the majority of these seed and any contaminating organisms were either killed by the agent, or, in the agar cultures, there was a growth of fungous hyphae from the discolored micropylar end. Such seed were necessarily discarded. The other seed mentioned had been stored under moist conditions, favoring the development of moulds and bacteria, and in no case was it possible to disinfect any reasonable percentage without injury in respect to

the capacity for vigorous germination. Similar observations upon the seed of certain grasses, sorghums, etc., which had undergone considerable heating during the curing process have led to the conviction that penetration of the seed by microörganisms, especially fungi, is not infrequent, and therefore a certain "purity" of the seed employed is requisite.

In table I are shown data obtained from the treatment of Canada field peas and corn with chlorinated lime, chlorinated potash, and chlorazene. In this case the first-mentioned disinfectant was more injurious to peas than in any other test made. The results with chlorinated potash were considered particularly good for the preliminary trials, while the results with chlorazene were disappointing.

Before proceeding further, tests were made, for comparison, with formalin and mercuric bichloride, and a second trial of chlorazene was included, as given in table II. The use of mercuric bichloride with such seed gave perfect disinfection, but these, as well as subsequent experiments, seemed to indicate that whenever this result was accomplished the injury to the seed was considerable. Various grades of alcohol, from 20 to 95 per cent, were also used in this series, the treatment

TABLE I

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 16 HOURS) BY CHLORINATED LIME, CHLORINATED POTASH (JAVEL WATER), AND CHLORAZENE. FINAL OBSERVATIONS AFTER 72 HOURS, ROOM TEMPERATURE

	Disinfectant	Treatment 3½ hours		Treatment ½ hour	
		% Germination	% Contamination	% Germination	% Contamination
Peas	25% chlor. lime	12	0	25	0
	15% chlor. lime	6	0	...	....
	25% chlor. potash	88	0	100	0
	10% chlor. potash	94	0	88	0
	4% chlorazene	0	4	0	0
	2% chlorazene	6	3–4	25	0
	1% chlorazene	42	0	87	0
Corn	25% chlor. lime	80	0	87	0
	15% chlor. lime	75	7	80	7
	25% chlor. potash	88	0	100	3
	10% chlor. potash	94	3–4	100	0
	4% chlorazene	94	0	100	11
	2% chlorazene	88	7	66	10
	1% chlorazene	75	0	87	7

TABLE II

DISINFECTION OF CANADA FIELD PEAS BY FORMALIN, MERCURIC BICHLORIDE, AND CHLORAZENE. FINAL OBSERVATIONS AFTER 96 HOURS, 26° C.

Disinfectant	Treatment 2 hours			Treatment 1 hour		
	% Germination	% Contamination	Condition	% Germination	% Contamination	Condition
* .2% formalin	77	50	Fair	93	10	Good
.2% formalin	70	66	Poor	83	43	Good
* .1% $HgCl_2$	16	0	Injured	24	0	Injured
.1% $HgCl_2$	47	0	Fair	76	0	Fair
*4% chlorazene	0	10	Injured	0	50	Fair
4% chlorazene	16	40	Fair	56	13	Fair
*2% chlorazene	33	6	Fair	43	6	Good
2% chlorazene	40	33	Good	63	6	Good
*1% chlorazene	86	20	Good	80	10	Good
1% chlorazene	83	30	Good	80	30	Good

* In these cases the seed were soaked for 16 hours prior to treatment, while in the other cases dry seed were treated.

being 5, 20, and 60 minutes. After 60 hours practically every seed was contaminated, so that the results are not tabulated.

In table III are given further results with chlorinated lime and potash. The intervals employed are too short for best results, so that the percentage of contamination runs high. Moreover, these experiments were made several months later than those included in tables I–II, and the seed were not so fresh. It seems clear, however, that short treatments are not satisfactory, and the value of longer intervals is particularly emphasized later.

A further extensive test of chlorinated lime, employing intervals of treatment up to 3 hours and using the freshest seed available, was made with the special view of determining the effect of the agent on the germination of the seed. The results are given in detail in table IV. The effectiveness of this disinfectant with corn and cucumber is clear. The percentage of germination with peas and radish is relatively low. This lot of radish seed proved difficult to sterilize, and the presence of a resistant organism spreading rapidly over the surface of the dishes tended to reduce the percentage of germination.

The test of sodium hypochlorite was made by preparing the concentrations indicated in table V from the solid substance.

TABLE III

DISINFECTION OF SEED BY CHLORINATED LIME AND CHLORINATED POTASH. FINAL OBSERVATIONS AFTER 96 HOURS

	Disinfectant	Treatment 1 hour			Treatment ½ hour		
		% Germination	% Contamination	Condition	% Germination	% Contamination	Condition
Peas	*20% chlor. lime	34	3	Fair	23	17	Fair
	20% chlor. lime	100	6	Good	87	0	Good
	*10% chlor. lime	37	13	Good	77	3	Fair
	10% chlor. lime	93	13	Good	93	0	Good
	* 5% chlor. lime	75	6	Fair	77	General	Fair
	5% chlor. lime	75	0	Good	100	0	Good
	*20% chlor. potash	80	20	.....	80	6	.....
	20% chlor. potash	75	13	.....	87	6	Good
	*10% chlor. potash	77	22	Good	77	50	Good
	10% chlor. potash	87	6	Good	93	6	Good
	* 5% chlor. potash	94	16	Good	77	50	Good
	5% chlor. potash	93	13	Good	87	0	Good
Corn	*20% chlor. lime	83	0	Fair	89	0	Good
	20% chlor. lime	88	20	Good	67	0	Good
	*10% chlor. lime	97	6	Good	67	0	Fair
	10% chlor. lime	95	6	Good	72	0	Fair
	* 5% chlor. lime	88	6	Good	78	10	Fair
	5% chlor. lime	88	0	Good	88	0	Fair
	*20% chlor. potash	75	3	Good	75	0	.....
	20% chlor. potash	67	6	Good	77	0	.....
	*10% chlor. potash	86	0	Good	67	General	Fair
	10% chlor. potash	83	13	Good	83	0	Fair
	* 5% chlor. potash	81	23	Good	75	General	Fair
	5% chlor. potash	83	6	Good	77	General	Fair

* In these cases the seed were soaked for four hours prior to treatment, while in the other cases dry seed were treated.

Although rather more erratic than results with other hypochlorites, the trial was of importance inasmuch as some dishes were free, or practically free, of contamination, and the percentage of germination relatively high. After further tests, however, it appeared that stronger solutions might be employed, and later a series of experiments was made in which this agent was compared with the potassium salt and with chlorazene, as shown by the data in table VI. These results are much more favorable than before for sodium hypochlorite; but the uniformly high percentage of germination, the good condition of the seedlings, and the freedom from contamina-

TABLE IV

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 4 HOURS) BY CHLORINATED LIME. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Concentration	Interval of treatment, hours	% Germination	% Contamination
Corn	10	2	72	0
	10	1	90	0
	10	½	90	0
	5	3	72	2
	5	2	84	0
	5	1	86	6
Peas	10	2	12	0
	10	1	24	0
	10	½	18	4
	5	3	38	0
	5	2	24	6
	5	1	32	4
Cucumber	10	2	84	0
	10	1	84	0
	10	½	68	2
	5	3	64	0
	5	2	82	8
	5	1	84	88
Radish	10	2	12	18
	10	1	30	14
	10	½	38	14
	5	3	4	56
	5	2	8	58
	5	1	28	24

tion (not perfect in the case of radish) where the potassium salt was employed, mark this as far more reliable under these conditions.

During the progress of this work other investigations were in progress by one of us and by graduate students, in which germinating seed free of contaminating organisms were required. The Javel treatment was employed, and with the consent of those who coöperated in this work, Dr. W. W. Bonns and Mr. T. Matsumoto, we are enabled to report the following facts: Of 500 seed of Canada field peas treated 3 hours with a 10 per cent solution the germination was 90 per cent and the contamination less than 1 per cent. In treating tobacco seed, 2- and 3-hour intervals proved inadequate; but a 4-hour interval with 12 per cent Javel water gave a contamination of less

TABLE V

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 4 HOURS AT 23° C.) BY SODIUM HYPOCHLORITE. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Concentration	Interval of treatment, hours	% Germination	% Contamination
Corn	10	2	96	2
	10	1	86	30
	10	½	92	4
	5	3	90	0
	5	2	92	0
	5	1	90	44
Peas	10	2	26	35
	10	1	30	96
	10	½	58	52
	5	3	40	58
	5	2	28	30
	5	1	30	20
Cucumber	10	2	88	50
	10	1	96	72
	10	½	92	100
	5	3	92	30
	5	2	82	14
	5	1	94	98
Radish	10	2	26	58
	10	1	18	10
	10	½	46	14
	5	3	46	6
	5	2	24	2
	5	1	26	4

than 2 per cent when 12 plates of about 30 seed each were sown. At the same time small quantities (2 dishes each) of lettuce, navy beans, and lima beans were similarly treated, with no contamination in any dish.

It was suggested that discontinuous disinfection might prove serviceable and practicable in work of this type, just as discontinuous sterilization by heat is so effective. Accordingly, experiments were arranged in which the dry seed were treated for 2 hours in deep Petri dishes with 20 per cent Javel water, then, after pouring this off, the seed were rinsed, and finally left in an incubator at room temperature for 2 days. The same treatment with rinsing was then repeated, and the seed placed for germination as in other cases. The results are shown in table VII, and these indicate complete disinfec-

TABLE VI

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 4 HOURS AT 23° C.) BY CHLORAZENE, CHLORINATED POTASH, AND SODIUM HYPOCHLORITE. FINAL OBSERVATIONS AFTER 96 HOURS

Disinfectant	Kind of seed	% Germination	% Contamination	Condition
1% chlorazene	Corn	100	50	Good
	Peas	26	100	Good
	Cucumber	100	...	Injured
	Radish	40	100	Good
20% chlor. potash	Corn	96	0	Good
	Peas	94	0	Good
	Cucumber	84	0	Good
	Radish	64	16	Good
20% sodium-hypochlorite	Corn	100	2	Good
	Peas	22	50	Injured
	Cucumber	96	0	Good
	Radish	12	0	Good

tion, as well as an unusually high percentage germination as compared with the usual percentage when the same seed are treated with any such disinfecting agent and placed directly on agar. The principle involved may possibly be widely applicable and deserves consideration in other work.

The results of similar tests with chlorinated lime, varying, however, the time intervals and the concentration of the disinfectant, are given in table VIII.

In addition to the results reported in full, extensive experiments were made with sodium chlorate and calcium sulphite. Concentrations of 10 and 5 per cent of the pure reagents were employed for various intervals, but in all cases 100 per cent

TABLE VII

DISINFECTION OF SEED BY 20 PER CENT CHLORINATED POTASH,—DISCONTINUOUS TREATMENT. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Germination	% Contamination	Condition
Corn.....	100	0	Fine
Peas.....	90	0	Good
Cucumber.....	100	0	Fine
Radish.....	50	20	Fair

TABLE VIII

DISINFECTION OF SEED BY CHLORINATED LIME,—DISCONTINUOUS TREATMENT. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Concentration	Interval of treatment, hours	% Germination	% Contamination
Corn	10	1	72	0
	*10	1	60	0
	*10	½	100	0
	5	2	56	28
	*5	2	100	0
	*5	1	96	0
Peas	10	1	20	8
	*10	1	52	0
	*10	½	24	50
	5	2	16	100
	*5	2	60	0
	*5	1	40	4
Cucumber	10	1	92	0
	*10	1	36	0
	*10	½	80	0
	5	2	60	100
	*5	2	72	12
	*5	1	100	0
Radish	10	1	0	12
	*10	1	8	16
	*10	½	14	100
	5	2	0	48
	*5	2	0	56
	*5	1	18	...

* In these cases the seed were soaked for 4 hours prior to treatment, while in the other cases dry seed were treated.

of contamination occurred, so that these and related substances were believed to be unsatisfactory for the purpose of this study.

Particular stress has been laid by de Zeeuw on the difficulty of properly washing off the disinfectant. He argues that quite commonly there may be transferred with the seed a sufficient amount of the disinfectant to insure antiseptic action during the germination of the seed on agar, but the unkilled germs may develop later—when the seed are transferred to final cultures. With the hypochlorites we have not been able to detect any such possibility, even when intentionally making the washing process less thorough than usual. Seed of beans,