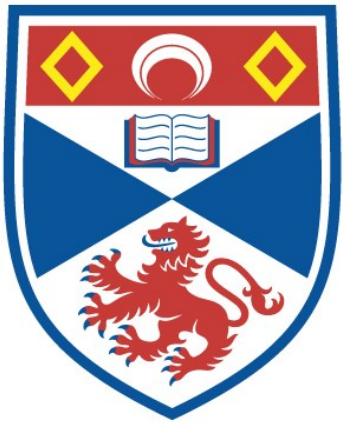
THE ROLE OF METABOLIC ADAPTATION IN THE AVOIDANCE OF SOAKING INJURY IN SEEDS

Colin R. Norton

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE ROLE OF METABOLIC ADAPTATION

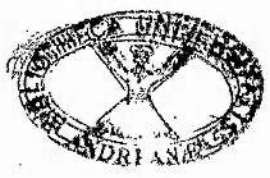
IN THE AVOIDANCE OF SOAKING INJURY

IN SEEDS

by

Colin R. Norton B.Sc., M.Sc.

A thesis submitted to the University of St.Andrews for the degree of Doctor of Philosophy, Department of Botany May 1975.



THE ROLE OF METABOLIC ADAPTATION IN THE AVOIDANCE OF SOAKING INJURY IN SEEDS.

Ph.D. thesis by Colin Norton presented to the University of St. Andrews 1975.

Seeds of a range of crop species were classified according to their ability to germinate after presowing soaking treatments of varying severity. The differences in soaking tolerance were then compared with changes in several metabolic parameters to determine the importance of metabolic adaptation in the avoidance of soaking injury in seeds.

After seed soaking treatments those species most susceptible to injury exhibited the highest respiration rates under nitrogen. At the same time the species with the highest respiration rates under nitrogen showed more rapid utilization of their seed reserves. This was demonstrated as a positive correlation of respiration rate under nitrogen against the fall in sucrose content of seeds on soaking.

A similar positive correlation of respiration under nitrogen with alcohol content and with fall in sucrose content on soaking seeds was found. However, when the malic acid content of seeds was assayed the reverse trend was shown, where malic acid was negatively correlated both with respiration under nitrogen and with the fall in sucrose content on

soaking seeds. Thus the changes in the alcohol and malic acid contents of seeds on soaking were negatively correlated with each other. Analysis of lactic acid content of soaked seeds was expressed as the percentage of the three anaerobic products measured (ie. alcohol plus malic acid plus lactic acid) and gave a significant negative correlation against respiration rate under nitrogen on soaking seeds.

Parallel studies of both alcohol and malic dehydrogenases were conducted after soaking seeds. The activity of alcohol dehydrogenase was highest in those species which were least tolerant of soaking injury, while the Michaelis constants for alcohol dehydrogenase with respect to acetaldehyde were low in species intolerant of soaking injury but higher in species tolerant of this treatment.

Enhanced metabolic damage was observed as a result of both high (30°C) and of low temperature (0°C) soaking treatments, but only in species which were normally susceptible to soaking injury. In studies on a single species (Pisum sativum) metabolic injury followed a similar course to the above studies in high but not in low temperature soaking treatments. Studies on the enzyme invertase showed grossly enhanced activity with a rise in temperature. This increased activity was also related to germination counts.

CAREER

I graduated from The University of Reading in July 1968 with an honours degree in Horticultural Science. From 1968 to 1969 I was Lecturer in Horticulture at The Askham Bryan College of Agriculture and Horticulture, where I was also Assistant Warden of Students.

In July 1970 I graduated from the University of Reading with the degree of M.Sc. in The Technology of Crop Protection.

I matriculated at The University of St.Andrews in October 1970 and was admitted as a candidate for the degree of Ph.D., while supported as a Research Assistant by The Agricultural Research Council.

On leaving The University of St.Andrews I was appointed to a Research Assistantship at The University of Dundee to work with Dr. J.Sprent on environmental effects on nodulation in legumes.

DECLARATION

I hereby declare that the following thesis is based upon work done by me, that the thesis is my own composition and that it has not previously been presented for a higher degree.

The research was carried out in The Department of Botany of The University of St.Andrews under the supervision of Dr.R.M.M. Crawford.

CERTIFICATE

I hereby certify that Colin R. Norton has been engaged upon research work for a minimum of nine terms under my supervision, and that he has fulfilled the conditions of Ordinance Number Twelve, and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS

I wish to record my gratitude to Dr.R.M.M. Crawford for supervising the work presented in this thesis and for his encouragement and enthusiasm throughout the project.

I appreciate the help given to me with the statistical analysis by Mr. A.Gordon of the Department of Statistics.

I am also pleased to acknowledge the Agricultural Research Council who supported me as a Research Assistant, and Professor Macdonald in whose department the work was carried out.

Especial thanks are due to my wife, Margaret, for her help in the preparation of this thesis.

CONTENTS

LIST OF SEEDS USED IN THE EXPERIMENTS

Broad bean - *Vicia faba* L. 'Aquadulce Longpod Bean'
(William Watt Seedsmen Ltd.)

Carrot - *Daucus carota* L. 'Stump Rooted Intermediate'
(William Watt Seedsmen Ltd.)

Lettuce - *Lactuca sativa* L. 'All the Year Round'
(William Watt Seedsmen Ltd.)

Maize - *Zea mays* L. 'Kelvedon Wonder'
(William Watt Seedsmen Ltd.)

Pea - *Pisum sativum* L. 'Gladstone'
'Meteor'
'Onward'
'Pioneer'
(William Watt Seedsmen Ltd.)

Radish - *Raphanus sativus* L. 'French Breakfast'
(William Watt Seedsmen Ltd.)

Rice - *Oryza sativa* L. 'Oeiras'
(Estacao Agronomica Nacional, Portugal).

PREFACE

Seeds of both wild and cultivated plants vary greatly in their sensitivity to soaking injury. In crops such as peas (Pisum sativum) poor field emergence is a constant and serious loss while in other crops such as rice (Oryza sativa) soaking of the seeds has no deleterious effects on germination capacity. In wild plants, species such as Juncus bufonius and Juncus tenuis seeds can lie dormant in wet peat or pond mud for years and are still capable of germination when exposed to air (Søhull, 1914). On the other hand, other wild species eg. Rhus glabra and Polygonum arifolium will not survive anaerobic conditions for more than a few days (Søhull, 1914). In spite of these fundamental differences in the germination capabilities of seeds, no detailed study has been made of the metabolic differences that may underlie their varying behaviours.

In higher plants, at the seedling and later stages of development, a number of metabolic differences has been detected between flood tolerant and flood intolerant species. There is a diversification of the end products of glycolysis (Crawford, 1972); for example in a study of the metabolism of roots of plants native to British bogs, those species which were able to withstand flooding

increased the malic acid content of their roots when flooded (Crawford and Tyler, 1969), while in a number of non flood tolerant plants flooding caused an increase in the rate of glycolysis with ethanol production (Crawford, 1966 and 1967). McManmon and Crawford (1971) report an apparent induction of alcohol dehydrogenase in flood intolerant species. They were also able to demonstrate the presence of 'malic enzyme' in flood intolerant species. This led them to speculate that the absence of the enzyme is necessary for malate to accumulate. The natural equilibrium for malic enzyme is to convert malate to pyruvate, which would then be easily metabolised to ethanol.

In these examples the situation is complex in that most species possess a number of mechanisms, both morphological and metabolic, which enable them to withstand periods of partial anaerobiosis. For a seedling, or grown plant, diffusion of oxygen from the shoot to the root is a common adaptation which reduces the dependence of the plant on metabolic adaptation to withstand oxygen deficits (Armstrong, 1964).

In the seed however, the situation does not have these complications as the onset of anaerobiosis affects the whole plant. Thus if seeds differ in their ability to survive this stress it must be

associated with differences in their metabolism.

This thesis therefore examines the ability of a number of seeds of different crop plants, to withstand periods of total or partial anaerobiosis and seeks to relate these varying germination tolerances to differences in metabolism of the seeds. In particular two appects of metabolism are investigated. The first, is the metabolic rate, as apparent in the respiration of the seeds. Usually the initial respiration in seeds is at least partially anaerobic due to the impermeability of the seed coat to oxygen (eg. Kolloffel. 1967 in Pisum sativum L.) but later there is a switch to aerobic respiration when the radicle ruptures the seed coat (Spragg and Yemm, 1959). When seeds are germinated under wet conditions the phenomenom of the reversal of the Pasteur Effect may cause an acceleration of glycolysis. Seeds also vary in the relative amounts of their products of glycolysis eg. alcohol accumulation (Doireau, 1969) or lactic acid accumulation (Sherwin and Simon, 1969). Respiration is therefore examined in seeds that are respiring in air and under nitrogen in order to determine the reaction of the respiration rate to the availability of oxygen. Secondly, the products of anaerobiosis in seeds tolerant of soaking are compared with those produced in seeds intolerant of this

treatment. This study parallels the investigations carried out by Crawford and coworkers (Crawford, 1966 and 1967; Crawford and Tyler, 1969; McManmon and Crawford,1971; Tyler and Crawford, 1970) into the study of flooding tolerance in higher plants, where differences were found in the nature and quantity of the products of anaerobiosis.

As changes in the amounts and rates of end product accumulation must reflect variations in enzyme properties, a number of comparative studies are made on the effects of soaking on the properties of a selected range of enzymes.

I. GERMINATION EXPERIMENTS

This study began by a repetition of the classical experiments of Morinaga (1926) in which seeds were classified according to their ability to germinate under water. This produses a simple dichotomy of soaking tolerant and soaking intolerant species. The effect of varying length of time of soaking on subsequent germination in moist sand was then examined. This extends the information from the first experiment by differentiating more finely between species of similar tolerance to soaking injury and allows a more accurate relative placing of seeds for their soaking tolerance.

Method

Thirty seeds of each of ten species were germinated on moist filter paper in petri dishes and further samples of the same species were submerged in beakers completely full of distilled water in incubators at 25°C (50 ml beakers for the smaller seeds and 250 ml beakers for the larger seeds). The percentage of seeds showing germination after one week was recorded, where germination was taken as the emergence of the radicle from the seed coat.

Results

The results are given in Table 1 as the percentage of seeds germinating under water and in controls on moist filter paper. A calculated probability for each pair of results, based on an amalgamation of soaking and control values, is also included. This is the probability of the observed ratio of control to under water germination, assuming that there was no effect due to soaking. Therefore, where the probability is less than $p = .05$ the theory that there is no effect due to soaking may be rejected (see statistical appendix). (Pages 232 to 234).

Radish, rice and carrot all germinated well underwater. As there was no significant difference between germination in controls and under water, these species may be described as soaking tolerant. Lettuce also germinated well under water (77%) but significantly less well than in the control ($p=.03$).

The remaining species (broad bean, Pioneer pea, Onward pea, Meteor pea, Gladstone pea and maize) failed to germinate under water. The results for these species are very highly significant with a probability of less than $p=.04 \times 10^{-10}$, and these species may be described as soaking intolerant.

Table 1.

Comparison of percentage germination under water with percentage germination on moist filter paper in air, after incubation for 7 days at 25°C (30 seeds of each species were used in each treatment).

Species	Control %germination	Underwater %germination	Probability of observed ratio of control: underwater germination arising by chance*.
Radish	96.7	96.7	.50
Rice	83.3	80.0	.49
Carrot	80.0	73.3	.38
Lettuce	96.7	76.7	.03
Broad bean	83.3	0	$.04 \times 10^{-10}$
Pioneer pea	86.7	0	$.04 \times 10^{-11}$
Onward pea	90.0	0	$.02 \times 10^{-13}$
Gladstone pea	96.7	0	$.03 \times 10^{-14}$
Maize	100	0	$.09 \times 10^{-16}$
Meteor pea	100	0	$.09 \times 10^{-16}$

* for details of the statistical test see appendix (p 232 to p 234).

The effect of soaking on germination is shown in Plates 1-3 for four species after 7 days soaking. From these plates it may be seen that cotyledon emergence was absent at this stage, but advanced radicle emergence was present in carrot (Plate 1) in contrast with radish (Plate 2) where only small radicles are visible, and maize (Plate 3) in which a swelling of the growing point was observed with rupturing of the seed coat in some cases. Maize seeds, even if left under water for several weeks showed no further occurrence of visible radicle development. In broad bean and the four pea cultivars there was no visible development at any stage and the seeds eventually decayed due to bacterial action.

Plate 1. Carrot seeds after germination for 7 days under water showing radicle development.(x1)

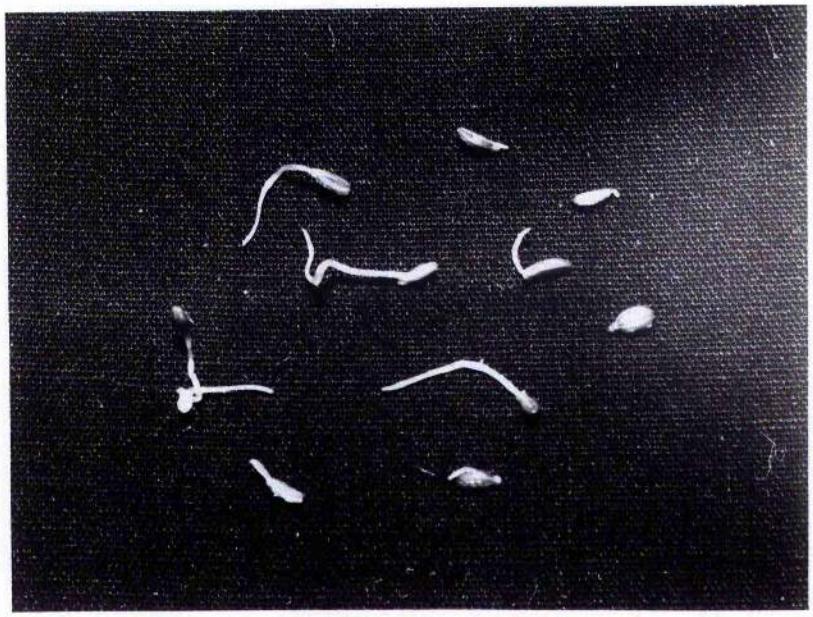


Plate 2. Radish seeds after germination under water for 7 days showing limited radicle development. (x3)

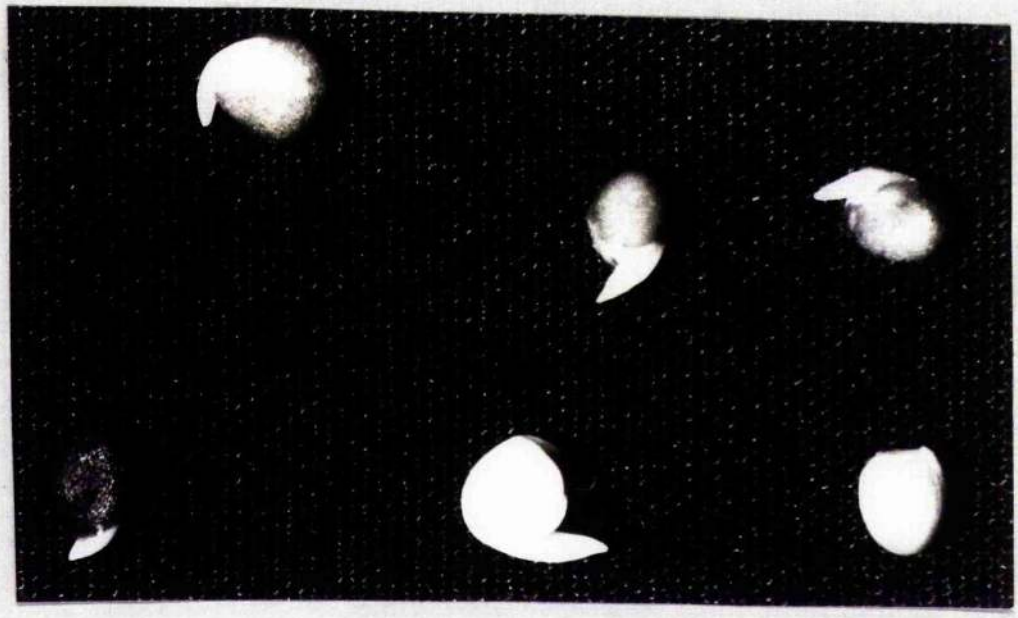


Plate 3. Maize seeds after germination under water for 7 days showing virtually no radicle development but rupturing of seed coats in some cases. (x.75)

The Relative Soaking Tolerance of Seeds of some Crop Species

In the preceding experiment seeds were germinated under partial anoxia (under water), but the effect of complete anoxia was not examined. A further experiment using anaerobe jars (GasPak) was therefore carried out to investigate the effect of complete anoxia on the germination of seeds. No germination was recorded in any of the treatments, which suggests that even the most soaking tolerant species have a minimal requirement for oxygen.

It is probable that in some species, anaerobic induced dormancy may result from soaking, and that the seeds will be able to recover on their return to an aerobic environment. The following experiment therefore examines the effect of a period of anaerobiosis (soaking treatments) prior to normal germination in aerobic conditions. This will also differentiate between species unable to withstand longer periods of anoxia.

Method

Thirty seeds of each of ten species were soaked for 0, 12, 24, 48, 72 or 96 hours and then germinated in trays of moist sand in a greenhouse at about 20°C. After fourteen days the final

germination count was taken and the heights of the individual seedlings were recorded.

Results

The results are given in Table 2 as the number of seeds germinated out of thirty for each treatment together with the correlation coefficient (r) for the number of seeds germinated against the number of hours of soaking (where germination was taken as the appearance of a shoot above the germination medium).

Rice, carrot, lettuce and radish did not show a significant correlation (using tables for calculated probability of r) for hours of soaking against number of seeds germinated; while Meteor and Pioneer peas gave significant negative correlations with probabilities of p=.05; broad bean gave a negative correlation p=.02; and Onward and Gladstone peas and maize all gave a negative correlation with p=.01 for chance occurrence of the observed correlation coefficient.

A regression analysis (Table 3) shows a significant decrease of germination with soaking in the same species. Table 4 shows the regression analyses which failed to reach significance. Figure 1 shows the fitted regression lines for those

species in which neither the regression nor the correlation coefficient for germination against duration of soaking was significant ie. soaking tolerant species. Figure 2 shows the soaking intolerant species in which a significant regression and correlation coefficient was shown.

Table 2.

Germination count at 14 days after varying duration of presoaking treatment of seeds.
(number germinated out of 30 when sown in trays of moist sand in the greenhouse).

Species	Duration of presoaking treatment in hours						Correlation coefficient hrs presoak: no. germinated	Probability of observed r by chance
	0	12	24	48	72	96		
Rice	12	29	24	28	21	29	+.456	N.S.
Lettuce	24	14	27	23	25	20	+.0632	N.S.
Carrot	13	25	20	21	17	11	-.440	N.S.
Radish	16	25	23	4	17	2	-.675	N.S.
Maize	27	28	27	25	18	14	-.949	.01
Meteor pea	22	20	22	16	0	0	-.896	.05
Pioneer pea	19	20	13	0	0	0	-.955	.05
Broad bean	27	30	26	27	15	10	-.910	.02
Onward pea	27	19	16	2	0	0	-.968	.01
Gladstone pea	24	20	19	2	0	0	-.960	.01

Table 3.

Regression analyses for number of seeds (out of 30) germinating after 14 days in moist sand against duration of presoaking treatment in hours.

Regression significant.

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Maize	Regression	150.321	1	150.321	36.415
	Residual	16.512	4	4.128	
	Total	166.833	5		(p=.01)
Meteor pea	Regression	275.862	1	275.862	12.163
	Residual	68.038	3	22.679	
	Total	344.000	4		(p=.05)
Pioneer pea	Regression	231.428	1	231.428	20.506
	Residual	22.572	2	11.286	
	Total	254.000	3		(p=.05)
Broad bean	Regression	266.477	1	266.477	19.539
	Residual	55.053	4	13.763	
	Total	321.500	5		(p=.025)
Onward pea	Regression	497.188	1	497.188	44.376
	Residual	33.612	3	11.204	
	Total	530.800	4		(p=.01)
Gladstone pea	Regression	457.284	1	457.284	35.434
	Residual	38.716	3	12.905	
	Total	496.000	4		(p=.01)

Table 4.

Regression analyses for number of seeds (out of 30) germinating after 14 days in moist sand against duration of presoaking treatment in hours.

Regression not significant.

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Rice	Regression	45.421	1	45.421	1.051
	Residual	173.312	4	43.328	
	Total	218.833	5		(N.S.)
Carrot	Regression	26.532	1	26.532	.962
	Residual	110.310	4	27.575	
	Total	136.833	5		(N.S.)
Lettuce	Regression	.462	1	.462	.0043
	Residual	106.407	4	106.407	
	Total	106.833	5		(N.S.)
Radish	Regression	208.426	1	208.426	3.347
	Residual	249.074	4	62.268	
	Total	457.500	5		(N.S.)

Figure 1.

Fitted regression line for number of seeds germinated (out of 30) against hours of presoaking treatment. Species tolerant of soaking (regression not significant).

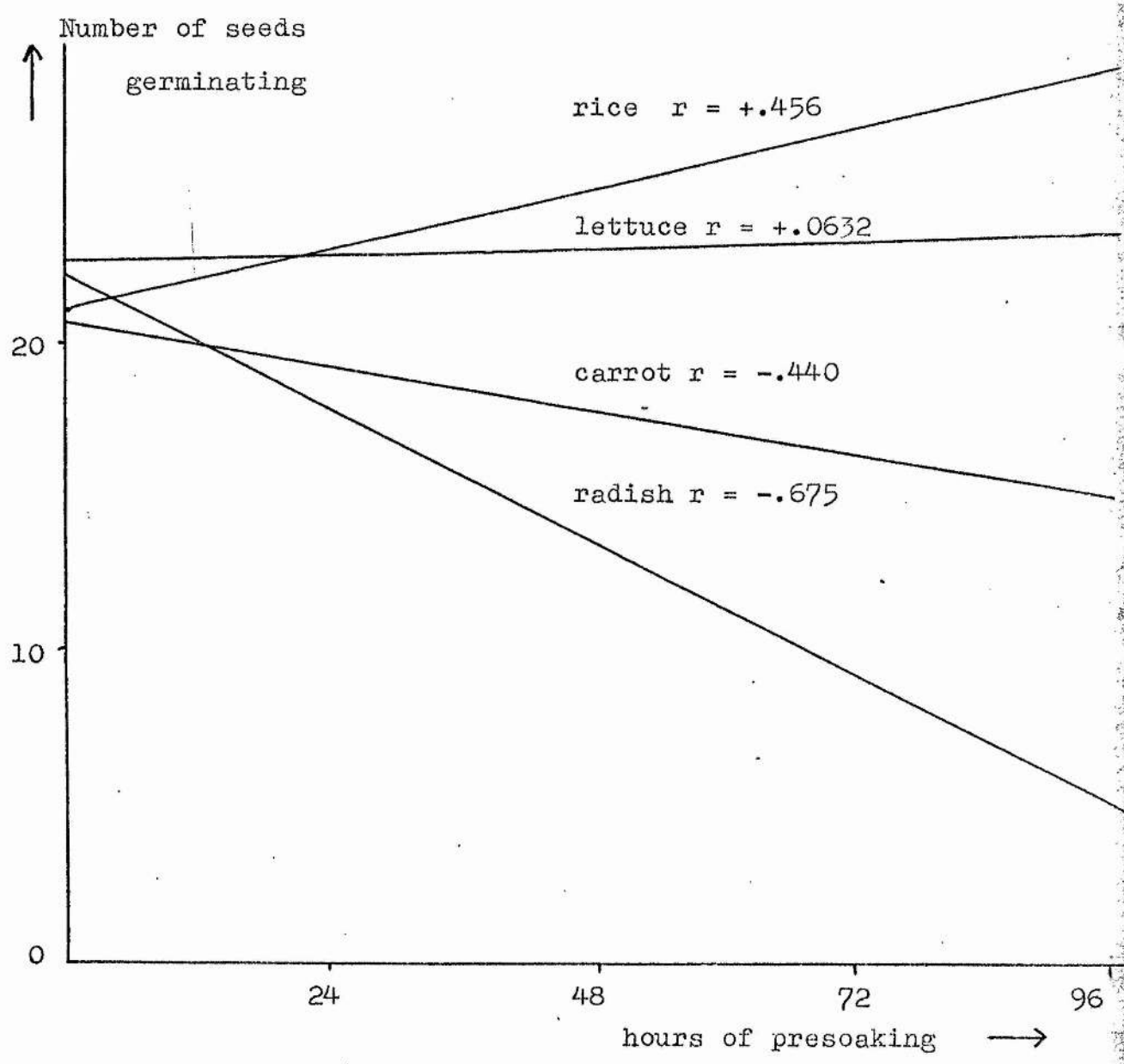
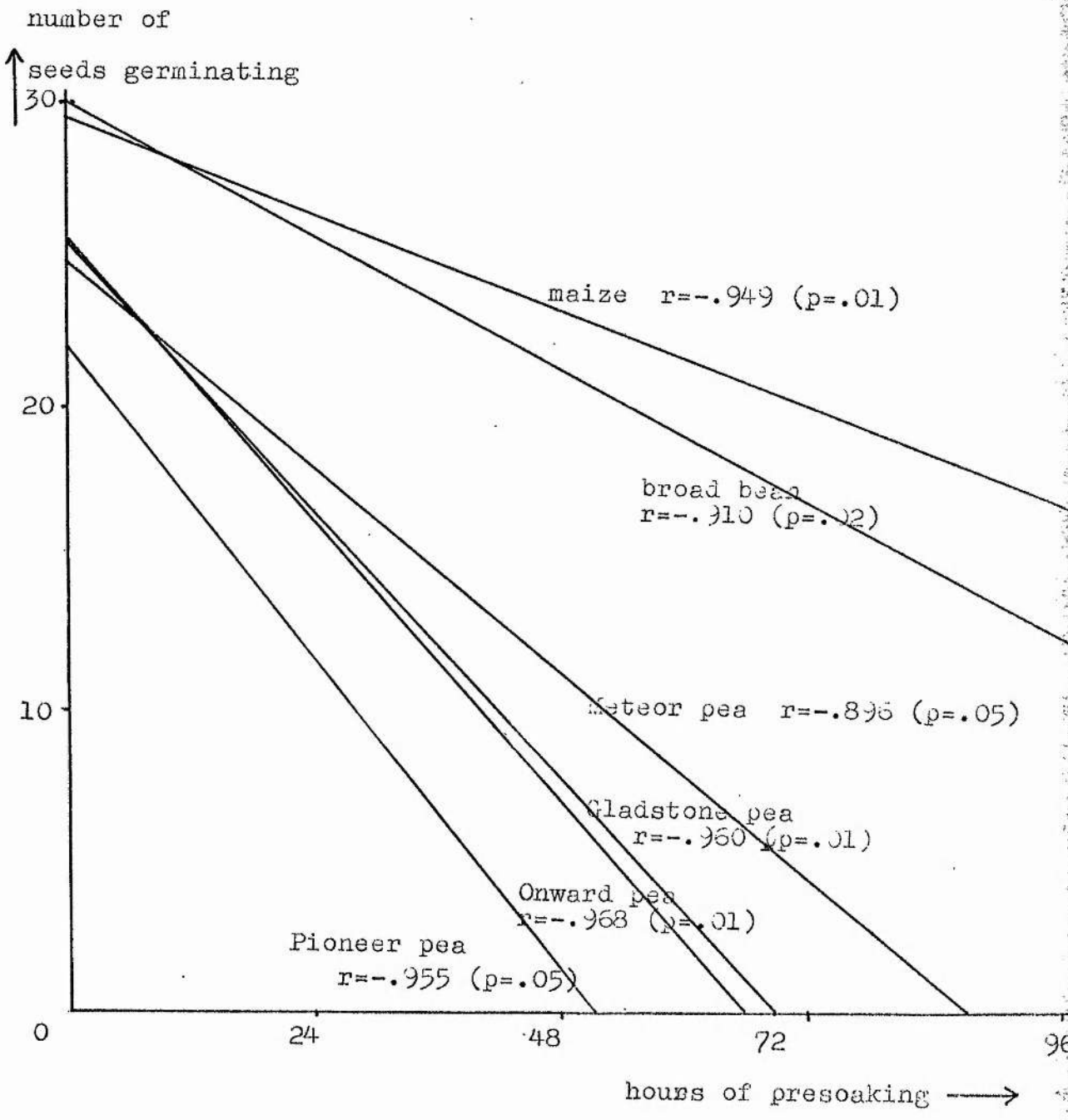


Figure 2.

Fitted regression lines for number of seeds germinating (out of 30) against hours of presoaking treatment. Species intolerant of soaking injury (regression significant)



Changes in morphology of seedlings after soaking seeds.

The foregoing analyses clearly divide the soaking tolerant from the soaking intolerant species but the division is based only upon the germination count. A further dimension may be added by an examination of the after effects on those seeds which successfully germinate after soaking compared with their controls. The height of seedlings was taken as a simple parameter to test this effect.

Results

Table 5 shows the results obtained for height (in cms) 14 days after sowing together with the correlation coefficient (r) for height against hours of presoaking treatment. Table 6 shows the equivalent regression analyses for height against duration of presoaking treatment for each species. A significant effect of soaking on height was not recorded in any case, although the correlation coefficient for broad bean fell only marginally short of significance. Since the heights of flood sensitive species could not be recorded in all treatments (due to failure of germination after longer soaking treatments) there were very few degrees of freedom for the above tests (2 to 3) and therefore the results should be interpreted with

caution.

Table 5 also shows the results of a students t test, which was used to compare the mean values after soaking with the control values in each species, but there is no clear trend in the results.

Table 5.

Mean heights (mms) of soaking sensitive species 14 days after sowing, with correaltion coefficient (r) for height against hours of presowing presoaking treatment. A students t test for height deviation from the control is also shown.

Species	Hours of presoaking treatment					Correlation coefficient height:hrs presoaking	Probability of r by chance
	0	12	24	48	72		
Meteor	68.67	29.24	29.75	56.00	-	-.0860	N.S.
S.D.	3.40	3.51	2.45	5.48			
Maize	60.37	69.66	98.89	111.60	42.63	-.0986	N.S.
S.D.	5.51	5.90	6.12	5.14	9.76		
Pioneer	65.74	25.21	51.25	-	-	-.351	N.S.
S.D.	4.56	2.91	5.81				
Broad bean	259.07	316.67	291.73	219.57	170.56	-.836	N.S. (for p=.05 r=.878)
S.D.	14.79	10.61	14.85	15.96	20.62		

Students t test for comparison with control

+ indicates a significant increase in height

- indicates a significant decrease in height

when compared with the control.

Table 6.

Regression analyses for height of seedlings (mm) at 14 days against hours of presoaking treatment in seeds sensitive to soaking injury.

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Maize	Regression	30.430	1	30.430	.029
	Residual	3156.948	3	1052.316	N.S.
	Total	3187.376	4		
Meteor pea	Regression	8.581	1	8.581	.015
	Residual	1150.279	2	575.140	N.S.
	Total	1158.860	3		
Pioneer pea	Regression	102.102	1	102.102	.138
	Residual	741.472	1	741.472	N.S.
	Total	843.574	2		
Broad bean	Regression	9440.286	1	9440.286	6.987
	Residual	4053.407	3	1351.136	N.S.
	Total	13493.693	4		

Induction of tissue damage on soaking seeds.

The previous two experiments in which the effect of germination on soaking characteristics was examined, showed that progressive soaking of sensitive species led to reduced germination. Recovery is not complete on return to aerobic conditions after soaking, and therefore the seed may be progressively damaged with soaking, and subsequently tissue degeneration may result. A tetrazolium stain is used to demonstrate this since it is known to react with live tissue to give a red colouration (due to a reaction with dehydrogenases which are present in all living plant tissue). If tissue degeneration is occurring progressively with the duration of soaking treatment in sensitive species, then in these species the reaction with tetrazolium should become less pronounced after longer soaking treatments.

Method

Seeds of each of ten species were soaked for 24, 48 and 96 hours in distilled water in an incubator at 25°C. They were then sectioned and immersed in a 1% solution of tetrazolium stain for 30 minutes after which they were examined under a microscope.

Results

No subjective measure was placed on the density of red colouration, but clear visual indication was given of loss of colouration in sensitive species with soaking. This is illustrated photographically in Plates 4 and 5 which show the effect of tetrazolium staining on two pea cultivars of differing sensitivity to soaking injury. Plate 4 shows the effect of a 24 hour soaking treatment in cv. Meteor (4 half seeds on left of picture) and in cv. Pioneer (4 half seeds on right). Both of these cultivars will germinate after a 24 hour soaking treatment. Plate 5 shows the effect of a 48 hour soaking treatment (Meteor on left and Pioneer on right). In this case cv. Pioneer shows a marked loss of colouration due to tissue death. A 48 hour soaking treatment prevented this cultivar from germinating while Meteor was able to do so.

Plate 4. Seeds of cvs. Meteor (4 half seeds on left) and Pioneer peas (4 half seeds on right) stained with tetrazolium stain after germination underwater for 24 hours. (x1)

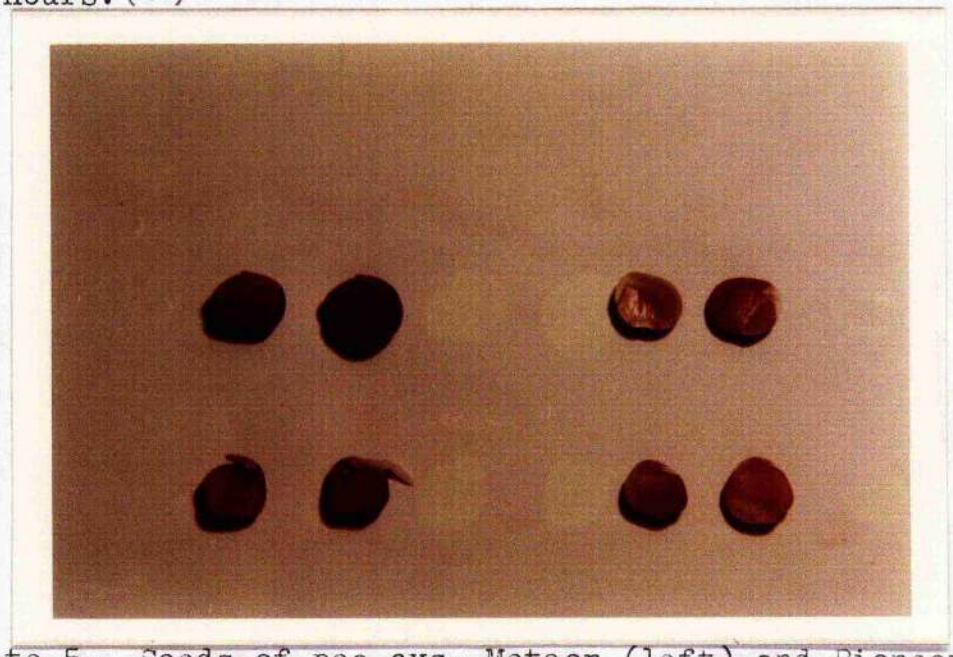
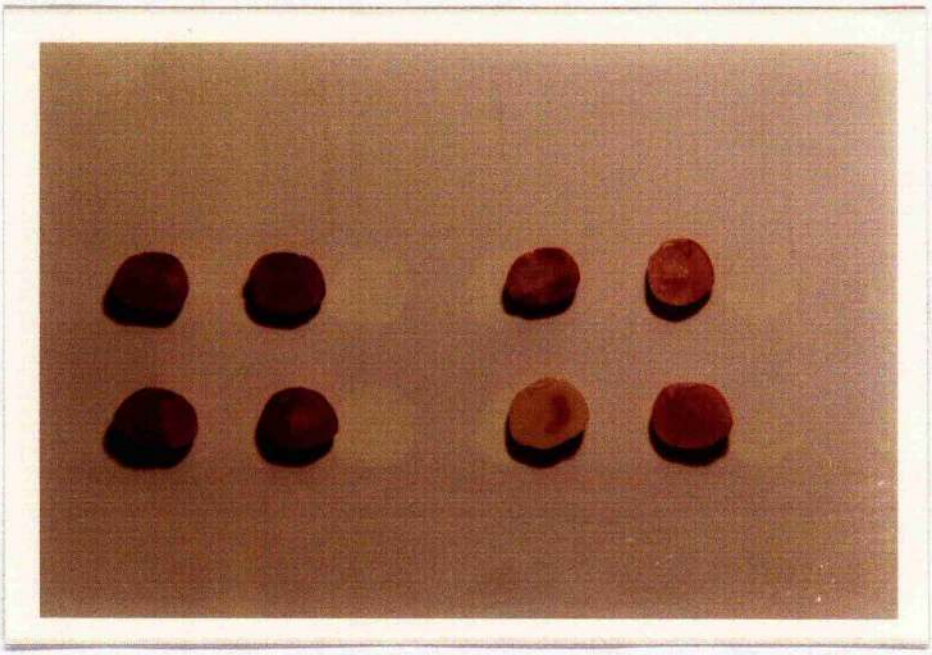


Plate 5. Seeds of pea cvs. Meteor (left) and Pioneer (right) stained with tetrazolium after germination underwater for 48 hours.(x1)



II. RESPIRATION AND SOAKING INJURY

This section examines the relationship between the soaking injury recorded in Chapter 1 and the respiration rates of seeds after similar soaking treatments. This has been approached from three aspects:-

1. A comparison of the rate of aerobic respiration with germination in conditions of partial anoxia.

When seeds are subjected to soaking, oxygen is able to diffuse into the seed only from the soak water and may therefore become limiting. Morinaga (1926) reported that a reduction in available oxygen during germination resulted in reduced germination in many species, but in a few others eg. Typha latifolia and Cynodon dactylon germination was increased with a reduction of available oxygen (8% oxygen optimal).

In the total absence of oxygen seeds are unable to germinate (Chapter 1, also James, 1953). Therefore, the small amount of oxygen dissolved in the water is likely to be important in determining the course and rate of respiration. Measurement of oxygen uptake compared with carbon dioxide evolution will reflect the potential for oxygen uptake, which is relevant to partial anoxia.

2. A comparison of respiration under nitrogen with germination ability under conditions of partial anoxia.

In the absence of oxygen plant tissues can continue to give off carbon dioxide (deSaussure, 1804) with the production of alcohol (Lechartier and Bellamy, 1869, 1872) and for each molecule of sugar consumed two molecules of alcohol and two molecules of carbon dioxide will be produced if the conversion is complete. This is the case with carrot tissue (James, 1971) which satisfies the Gay Lussac equation to within 1%.

$$C_6H_{12}O_6 \rightleftharpoons 2C_2H_5OH + 2CO_2$$

However, alcohol is not the sole product of anaerobic respiration in plant tissues but it is often the major conponent.

During temporary anaerobiosis lactic acid accumulates in many animal tissues (eg. kidney cells). It is not toxic and can be remetabolised when oxygen is available. In plants lactic acid accumulation is less common, but in potatoes for example, half of the product of anaerobic respiration is lactate (James, 1971). Since alcohol and lactate are the two most frequently reported anaerobic products in plants, it is likely that those seeds which are able to accumulate lactate in preference to alcohol

may have a higher tolerance of anaerobic respiration, in that lactate may be remetabolised to repay the oxygen debt while alcohol is normally irreversibly formed (James, 1971).

A comparison of the levels of respiration under nitrogen will, therefore, give a measure of the activity of the seed after soaking and of the possible accumulation of toxic end products. (This will not give a measure of non toxic end products, such as lactate, because no carbon dioxide is liberated during its formation).

3. An examination of changes in the rate of glycolysis after germination under partial anoxia.

As already discussed not only is the rate of end product accumulation important, but the change in rate is also important. This is because metabolic rates alone do not indicate the nature of a plant response to any particular environmental stress. However, when the change of rate of any particular reaction is noted, then this can be related to the effect of the environmental change (in this case partial anoxia) on the process under investigation.

In a study of carbon dioxide evolution, James (1971) was able to show that carrot tissues under anaerobiosis (under nitrogen) increased

carbon dioxide emission with alcohol accumulation, while carbon dioxide emission in potatoes fell under anaerobiosis, with lactate accumulation. In potato there followed a period of rapid carbon dioxide emission on return to air, as the lactate was remetabolised, but in carrot there was no such increase.

It is therefore important to determine whether seeds, when soaked, accumulate alcohol or non toxic end products, or even slow their respiration rate to avoid accumulation of toxic products in too high a concentration.

Method - Changes in Pisum sativum L and in rice

A varying number of seeds, depending on seed type, were taken after soaking treatments of 3, 6, 12, 24 or 48 hours at 25°C and their respiration rates were measured using a Warburg apparatus. The rate of anaerobic respiration was measured after gassing the flasks for ten minutes with nitrogen in order to simulate anaerobic conditions.

Results

Tables 7, 8 and 9 show the resuts obtained. A regression analysis was carried out (see Tables 66-68 statistical appendix) for the respiratory quotient (RQ), oxygen uptake ($\mathcal{Q}O_2$), carbon dioxide evolution ($\mathcal{Q}CO_2$) and respiration under nitrogen ($\mathcal{Q}CO_2{}^n$ hr^{-1})

with duration of soaking treatment and those analyses demonstrating significant changes are shown in figures 3, 4 and 5.

Anaerobic respiration rate ($\mathcal{Q}CO_2^{n}$ hr^{-1}) gave a significant negative linear correlation with duration of soaking treatment in Pioneer pea ($p=.01$), while in Meteor pea $\mathcal{Q}CO_2^{n}hr^{-1}$ was positively correlated with duration of soaking treatment ($p=.05$). In rice both $\mathcal{Q}O_2$ and $\mathcal{Q}CO_2$ were positively correlated with duration of soaking treatment ($p=.01$ and $p=.05$ respectively).

Table 7. Respiration rates (μl mg dry weight^{-1}hr^{-1}) of Pioneer pea seeds after soaking in water or germinated on moist filter paper for varying lengths of time.

Duration of soaking	QO_2	QCO_2	RQ	$QCO_2{}^{n}hr^{-1}$
3 hours	.240	.426	1.775	.666
S.D.	.0308	.0664	.356	.0497
6 hours	.411	.469	1.141	.685
S.D.	.0159	.0763	.211	.0134
12 hours	.378	.555	1.468	.595
S.D.	.00263	.0430	.104	.0281
24 hours	.339	.395	1.165	.569
S.D.	.0170	.00999	.0480	.0397
48 hours	.314	.367	1.168	.468
S.D.	.0631	.0527	.0996	.157
Duration of germination on moist filter paper				
12 hours	.332	.358	1.078	.350
S.D.	.0315	.0292	.0558	.123
24 hours	.352	.303	.861	.490
S.D.	.0719	.0855	.146	.0306

Values in the body of the table are each means of four replicates.

Table 8. Respiration rates (μl mg dry weight^{-1}hr^{-1}) of Meteor pea seeds after soaking in water or germinated on moist filter paper for varying lengths of time.

Duration of soaking	QO_2	QCO_2	RQ	$QCO_2{}^n$ hr^{-1}
3 hours	.229	.195	.851	.317
S.D.	.0316	.00570	.0528	.0354
6 hours	.292	.291	.997	.331
S.D.	.0331	.0509	.286	.0102
12 hours	.310	.351	1.132	.403
S.D.	.0287	.0320	.0461	.0234
24 hours	.298	.407	1.366	.445
S.D.	.0430	.0626	.0447	.0253
48 hours	.329	.375	1.140	.476
S.D.	.0310	.0500	.163	.0265
Duration of germination on moist filter paper				
12 hours	.185	.151	.816	.217
S.D.	.0642	.0904	.276	.0592
24 hours	.448	.322	.719	.398
S.D.	.118	.109	.128	.0715

Each value in the body of the table is a mean of four replicates.

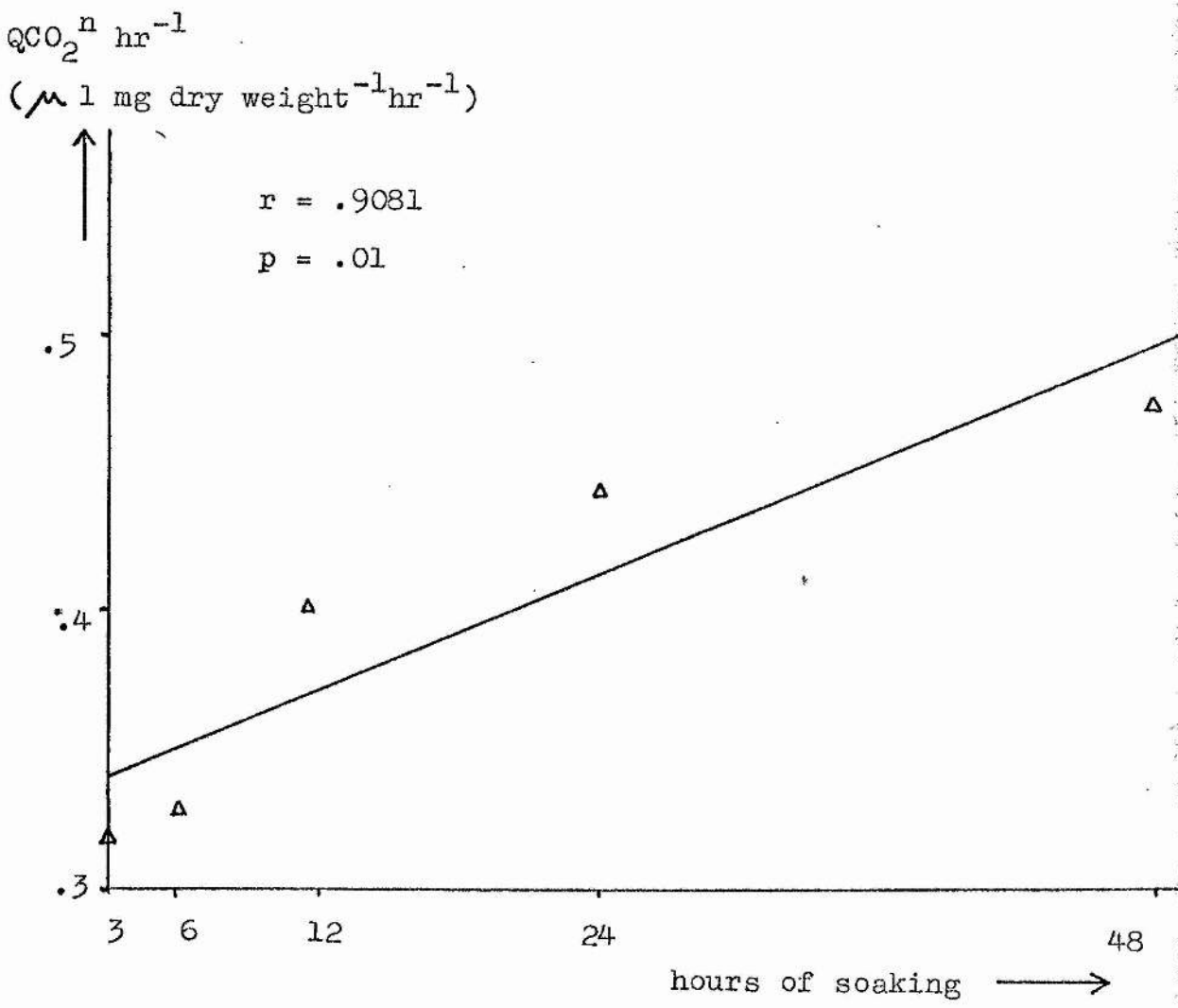
Table 9. Respiration rates (μl mg dry weight^{-1}hr^{-1}) of rice seeds after soaking in water or germinated on moist filter paper for varying lengths of time.

Duration of soaking	$\mathcal{Q}O_2$	$\mathcal{Q}CO_2$	RQ	$\mathcal{Q}CO_2{}^n$hr^{-1}
3 hours	.0127	.0103	.811	.0510
S.D.	.00286	.00386	.136	.00312
6 hours	.0125	.0129	1.032	.0184
S.D.	.00383	.00500	.123	.00892
12 hours	.0132	.0557	4.220	.0603
S.D.	.00615	.0110	2.367	.00770
24 hours	.0170	.101	5.941	.103
S.D.	.000577	.0272	1.477	.00317
48 hours	.0270	.108	4.000	.113
S.D.	.00666	.0262	1.944	.0127
Duration of germination on moist filter paper				
12 hours	.00748	.0232	3.102	.0363
S.D.	.00151	.00691	.115	.0111
24 hours	.0822	.101	1.229	.0458
S.D.	.0177	.0225	.0312	.0142

Each value in the body of the table is a mean of four replicates.

Figure 3.

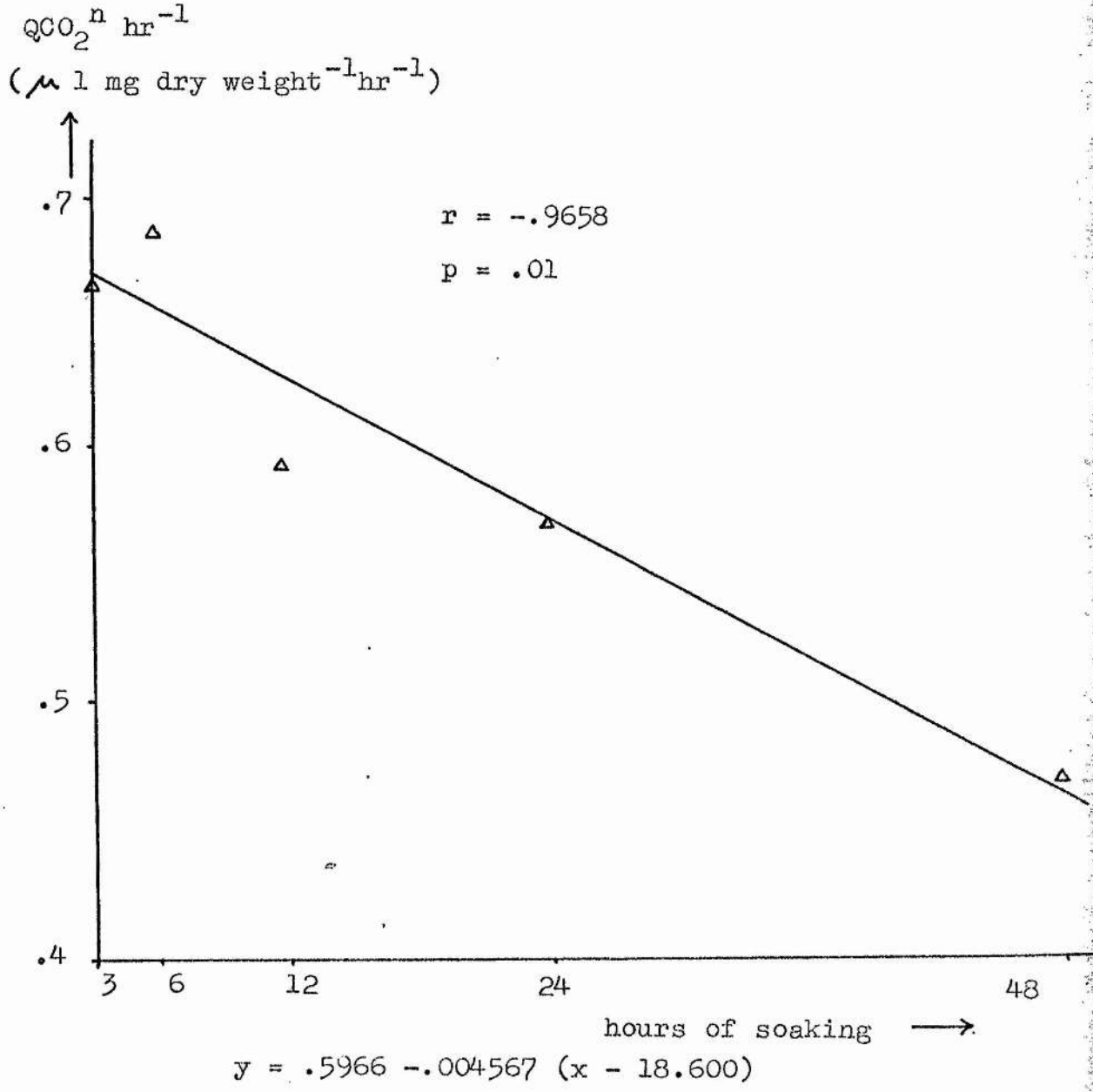
Fitted regression line for increase in anærobic respiration rate under nitrogen (μl CO_2 mg dry wight^{-1} hr^{-1}) with duration of soaking treatment in Meteor pea seeds



$$y = .3944 + .003451\ (x - 18.600)$$

Figure 4.

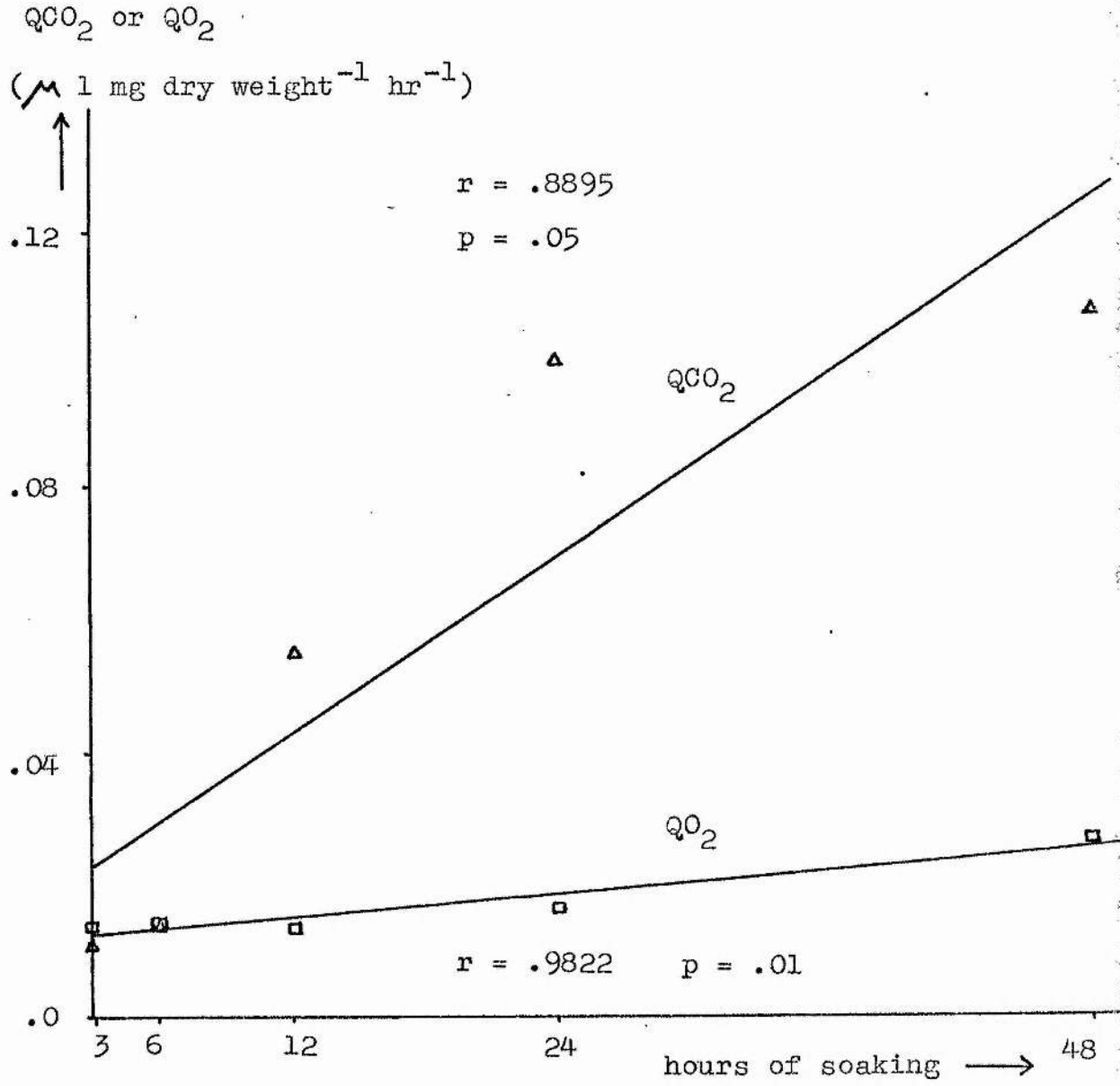
Fitted regression line for decrease in anaerobic respiration rate under nitrogen (μl mg dry weight^{-1} hr^{-1}) with duration of soaking treatment in Pioneer pea seeds.



$y = .5966 - .004567 (x - 18.600)$

Figure 5.

Fitted regression lines for increase in carbon dioxide evolution and oxygen uptake (µl mg dry weight^{-1}hr^{-1}) with duration of soaking treatment in rice seeds.



QO_2 fitted regression line $y = .01648 + .0003310(x - 18.600)$

QCO_2 $y = .05758 + .002262(x - 18.600)$

The mean overall values after soaking for 3 to 48 hours for QO_2, QCO_2, RQ and QCO_2^n were compared between species. All interspecies comparisons were significantly different (Table 10).

Except for RQ values, Pioneer pea gave the highest rates for all the respiration measurements, followed by Meteor pea and the lowest values were recorded for rice. However, rice gave the highest RQ value, then Pioneer pea and least in Meteor.

A comparison of air and water treatments was conducted overall for the 12 and 24 hour soaking treatments. The results are presented in Table 11. In Meteor pea, Pioneer pea and rice, there was a significant increase in RQ on soaking, and in Meteor pea and Pioneer pea there was a significant increase in QCO_2. Pioneer pea also showed a significant increase in QCO_2^n on soaking.

Table 10. Mean respiration rates (μl mg dry weight^{-1} hr^{-1}) for 3 to 48 hour soaking treatments of Pioneer pea, Meteor pea and rice seeds.

Species	QO_2	QCO_2	RQ	QCO_2^n
Meteor pea	.2916	.3238	1.0972	.3944
Pioneer pea	.3364	.4424	1.3434	.5966
rice	.01648	.05760	3.2010	.06914

(Probability of observed difference in column means arising by chance p=.001 an all cases).

Table 11.

Comparison of respiration rates (μl mg dry weight^{-1} hr^{-1}) of seeds after soaking underwater with respiration rates of seeds germinated on moist filter paper in Meteor pea, Pioneer pea and in rice.

Species	QO_2	QCO_2	RQ	QCO_2^n
Meteor control	.3165	.2365	.7675	.3075
underwater	.3040	.3790	1.2490	.4040
p	N.S.	.02	.001	N.S.
Pioneer control	.3420	.3305	.9695	.4200
underwater	.3585	.4750	1.1565	.5820
p	N.S.	.001	.01	.002
rice control	.0411	.0621	2.166	.0411
underwater	.0151	.0784	5.081	.0817
p	.001	N.S.	.02	N.S.

All values within the table of means are means of both 12 and 24 hour soaking treatments. Probability (p) is based on a students t test for chance occurrence of the difference between control and underwater treatments.

Changes in respiration rate on soaking seeds of a wider range of species.

Similar experiments were carried out for a wider range of seed types, but with fewer treatments. The results are shown in Tables 12 (soaking tolerant species) and 13 (soaking susceptible species) with a students t test for the difference between seeds soaked underwater for 24 hours and seeds germinated on moist filter paper for 24 hours for each species (Pioneer pea, Meteor pea, rice included for comparison). Rice, radish, maize and broad bean showed significantly reduced oxygen uptake while carrot showed a significant increase. Carrot and Meteor pea showed an increase in carbon dioxide evolution whereas radish decreased carbon dioxide evolution significantly. Rice, broad bean and Meteor pea gave an increase in RQ. Rice, maize and broad bean showed a significant increase in anaerobic respiration rate under nitrogen while carrot and radish decreased respiration rates under nitrogen significantly on soaking. Table 14 shows the relationship between respiration under nitrogen and germination ability underwater.

Table 12.

Comparison of respiration rates (μl mg dry weight^{-1} hr^{-1}) between seeds soaked for 24 hours underwater and seeds germinated for 24 hours on moist filter paper in a range of species.

Species tolerant of soaking injury.

Species	QO_2	QCO_2	RQ	QCO_2^n
rice control	.0822	.101	1.229	.0458
underwater	.0170	.101	5.941	.103
p	.02	N.S.	.02	.02
radish control	.465	.361	.776	.172
underwater	.290	.245	.845	.0616
p	.05	.05	N.S.	.001
lettuce control	.461	.307	.666	.176
underwater	.404	.264	.653	.199
p	N.S.	N.S.	N.S.	N.S.
carrot control	.225	.147	.653	.0677
underwater	.489	.360	.736	.0263
p	.001	.02	N.S.	.05

All values in the table are means of four replicates. The probability (p) is the probability of chance observation of the difference between the control value and the respiration rate for underwater germination.

Table 13.

Comparison of respiration rates (μl mg dry weight^{-1} hr^{-1}) between seeds soaked for 24 hours underwater and seeds germinated for 24 hours on moist filter paper in a range of species.

Species intolerant of soaking injury.

Species	QO_2	QCO_2	RQ	QCO_2^n
maize control	.144	.199	1.382	.144
underwater	.0536	.186	3.470	.240
p	.001	N.S.	N.S.	.02
broad bean control	.315	.190	.633	.164
underwater	.254	.290	1.142	.269
p	.02	N.S.	.05	.05
Meteor pea control	.448	.322	.719	.398
underwater	.298	1.407	1.366	.455
p	N.S.	.001	.02	N.S.
Pioneer control	.352	.303	.861	.490
underwater	.339	.395	1.165	.569
p	N.S.	N.S.	N.S.	N.S.

All the values in the table are means of four replicates. The probability (p) is the probability of chance observation of the difference between the control value and the respiration rate for underwater germination.

Table 14.

The relationship between the rate of respiration under nitrogen (μl mg dry weight^{-1}hr^{-1}) and germination ability underwater in the seeds of some crop species.

Species	Respiration under nitrogen(l mg dry weight^{-1}hr^{-1})	Germination under water
Pioneer pea	.569	none ($p=.04 \times 10^{-10}$)
Meteor pea	.445	none ($p=.09 \times 10^{-16}$)
broad bean	.269	none ($p=.04 \times 10^{-10}$)
maize	.240	none ($p=.09 \times 10^{-16}$) but slight radicle emergence
lettuce	.199	germinates slightly less well underwater($p=.03$)
rice	.103	germinates equally well ($p=.49$)
radish	.0616	germinates equally well ($p=.50$)
carrot	.0263	germinates equally well ($p=.38$)

All respiration rates are means of four values.
The bracketed probability figures are the probabilities of germination underwater based on the binomial used in Chapter 1.

III. SOAKING AND SUGAR METABOLISM IN SEEDS

When seeds germinate respiratory energy is provided from the breakdown of carbohydrate reserves within the seed. Since carbohydrates are the main storage products in seeds a study of their breakdown will give an indication of the major metabolic changes in the seed.

During germination, starch is usually converted by the enzyme amylase to sugars, the levels of which normally increase during germination (in castor beans, Yamada 1955; in wheat, Yocum, 1925; in barley, McLeod, 1957), prior to being respired. Morohashi and Shimokoriyama (1972) found the main sugars to be sucrose, fructose and glucose in chromatographic extracts of Phaseolus mungo and they reported that sucrose was the main free sugar.

However, McLeod observed no increase in sugar in barley when given a 24 hour soaking treatment, while Morohashi and Shimokoriyama (1972) demonstrated a decline in sucrose levels during the first nine hours of germination in Phaseolus. In both these cases there is a period of at least partial anaerobiosis. A probable explanation for such a fall lies in the fact that the energy yield per molecule of sugar

is far less for anaerobic respiration and therefore more sugar is likely to be consumed to meet the energy requirement of the seed.

This section, therefore, examines the effect of soaking on seeds as shown by changes in both non reducing (sucrose) and in reducing sugars (fructose and glucose) in relation to germination and respiration rates of seeds after soaking.

Method

A known weight of seed of each of ten crop species was soaked for 0, 12, 24, 48 or 96 hours in 50 ml beakers completely full of distilled water at 25°C. The seeds were then extracted in distilled water, passed through muslin and centrifuged at 7,000 rpm for 30 minutes. Samples taken both before and after inversion with the enzyme invertase (in a shaker bath at 25°C for two hours) were titrated against known thiosulphate (as detailed in the appendix).

Results

Table 15 gives the results as mg sucrose and as mg fructose + glucose per g dry weight seed with standard deviations based on three replicates for soaking tolerant species. Table 16 gives the results for soaking sensitive species.

Figure 7 shows these results graphically for sucrose in soaking tolerant species and in Figure 8 for soaking intolerant species.

Regression analyses were carried out for fructose + glucose and for sucrose (mg g^{-1} dry weight seed) against duration of presoaking treatment, and the results of an F test on each species regression is given in Table 17, for soaking tolerant species, and in Table 18 for soaking intolerant species and in Table 19 for four pea cultivars. Only broad bean, Meteor pea, Gladstone pea and maize showed a significant linear fall in sucrose with increase in duration of soaking treatment. Table 20 shows the overall change in sugars as mg g^{-1} dry weight seed hr^{-1}, calculated from their respective regression equations for sugar mg g^{-1} dry weight seeds against duration of presoaking treatment.

Table 15. Changes in mg fructose + glucose and in sucrose (mg g^{-1} dry weight seed) after 0 to 96 hour presoaking treatments in the seeds of some crop species.

Species tolerant of soaking injury.

Species	Hours of presoaking treatment				
	0	12	24	48	96
rice fr/gl	.441	2.31	4.14	2.37	3.42
S.D.	.0180	.924	.0367	.600	.0367
rice sucrose	8.56	1.67	.757	.760	.270
S.D.	.335	.108	.274	.282	.117
lettuce fr/gl	24.84	36.00	47.16	28.80	28.44
S.D.	2.16	1.90	.952	1.30	1.57
lettuce sucrose	20.16	12.24	5.76	17.28	4.32
S.D.	4.01	2.88	3.20	2.49	1.08
carrot fr/gl	14.40	25.02	25.56	6.84	10.44
S.D.	.476	1.18	.900	.649	1.72
carrot sucrose	6.03	4.14	4.32	2.43	7.92
S.D.	.800	.952	.994	.270	1.72
radish fr/gl	13.50	3.08	23.13	11.61	17.01
S.D.	.412	.0312	.090	.468	.412
radish sucrose	22.32	3.51	63.27	4.77	20.96
S.D.	1.362	.195	2.66	.630	1.63

Standard deviations (S.D.) are based on three replicates.
fr/gl = sum of fructose and glucose in mg g^{-1} seed.

Table 16. Changes in mg fructose + glucose and in sucrose (mg g^{-1} dry weight seed) after 0 to 96 hour presoaking treatments in the seeds of some crop species.

Species intolerant of soaking injury.

Species	Hours of presoaking treatment				
	0	12	24	48	96
maize fr/gl	8.12	12.65	10.94	8.06	12.42
S.D.	.065	.217	.157	.367	.489
maize sucrose	21.73	24.16	22.27	18.31	7.18
S.D.	.692	.941	2.67	.219	.163
Gladstone fr/gl	5.94	2.18	7.67	5.18	11.56
S.D.	.562	.255	.064	.286	.061
Gladstone sucrose	37.26	29.50	31.66	19.08	11.34
S.D.	1.09	.079	3.48	2.36	.590
Meteor fr/gl	.648	1.91	5.02	3.47	6.73
S.D.	.083	.065	.567	.312	.234
Meteor sucrose	25.81	26.80	19.33	9.13	7.76
S.D.	.235	.693	.887	.601	.315
Onward fr/gl	5.00	3.98	5.99	2.57	5.56
S.D.	.603	.199	.221	.390	.082
Onward sucrose	33.57	35.80	31.36	8.42	10.91
S.D.	.737	.434	1.67	.972	.411
Pioneer fr/gl	1.44	3.11	7.60	4.37	6.84
S.D.	.018	.036	.097	.204	.176
Pioneer sucrose	29.25	28.93	32.54	14.53	12.53
S.D.	1.93	6.61	6.40	.809	4.11
broad bean fr/gl	5.14	6.55	5.60	2.07	3.21
S.D.	.098	.148	1.454	.393	.370
broad bean sucrose	28.70	24.32	20.41	16.20	14.01
S.D.	.972	.872	1.81	.686	1.02

Standard deviations (S.D.) are based on three replicates.

fr/gl = sum of fructose and glucose mg g^{-1} seed.(dry weight).

Table 17. Regression analyses for changes in sugar (mg g^{-1} dry weight seed) with duration of presoaking treatment (0 to 96 hours) in the seeds of some crop species which are tolerant of soaking injury.

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
rice fr/gl	Regression	2.2029	1	2.2029	1.176
	Residual	5.6185	3	1.8728	
	Total	7.8214	4		N.S.
rice sucrose	Regression	19.1476	1	19.1476	1.998
	Residual	29.2505	3	9.7502	
	Total	48.3981	4		N.S.
lettuce fr/gl	Regression	12.8596	1	12.8596	.121
	Residual	301.654	3	100.551	
	Total	314.514	4		N.S.
lettuce sucrose	Regression	67.185	1	67.185	1.609
	Residual	125.246	3	41.749	
	Total	192.431	4		N.S.
carrot fr/gl	Regression	89.281	1	89.281	1.340
	Residual	199.830	3	66.610	N.S.
	Total	217.110	4		
carrot sucrose	Regression	.01089	1	.01089	.00188
	Residual	17.378	3	5.7927	
	Total	17.389	4		N.S.
radish fr/gl	Regression	18.055	1	18.055	.272
	Residual	199.055	3	66.352	
	Total	217.110	4		N.S.
radish sucrose	Regression	19.072	1	19.072	.0246
	Residual	2319.892	3	773.297	
	Total	2338.909	4		N.S.

fr/gl = summation of fructose and glucose

Table 18.

Regression analyses for changes in sugar (mg g^{-1} dry weight seed) with duration of presoaking treatment (0 to 96 hours) in the seeds of some crop species which are sensitive to soaking injury.

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
maize fr/gl	Regression	2.2877	1	2.2877	.385
	Residual	17.8228	3	5.9409	
	Total	20.1105	4		N.S.
maize sucrose	Regression	166.2930	1	166.293	27.293
	Residual	18.2786	3	6.0929	
	Total	47.6800	4		p=.025
broad bean fr/gl	Regression	6.4000	1	6.4000	2.747
	Residual	6.9901	3	2.330	
	Total	13.3901	4		N.S.
broad bean sucr.	Regression	118.680	1	118.680	2.747
	Residual	23.5107	3	7.837	
	Total	142.191	4		p=.05

fr/gl = summation of fructose and glucose

Table 19. Regression analyses for changes in sugar (mg g^{-1} dry weight seed) with duration of presoaking treatment (0 to 96 hours) in the seeds of four pea cultivars which are sensitive to soaking injury.

Cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Gladstone fr/gl	Regression	27.477	1	27.477	4.070
	Residual	20.233	3	6.744	
	Total	47.680	4		N.S.
Gladst. sucrose	Regression	401.069	1	401.069	36.980
	Residual	32.537	3	10.846	
	Total	433.606	4		p=.01
Meteor fr/gl	Regression	17.350	1	17.350	8.609
	Residual	6.047	3	2.015	
	Total	23.396	4		N.S.
Meteor sucrose	Regression	262.369	1	262.369	12.854
	Residual	61.234	3	20.411	
	Total	323.603	4		p=.05
Onward fr/gl	Regression	.0488	1	.0488	.0192
	Residual	7.626	3	2.542	N.S.
	Total	7.577	4		
Onward sucrose	Regression	494.884	1	494.884	7.275
	Residual	204.069	3	68.023	
	Total	698.953	4		N.S.
Pioneer fr/gl	Regression	11.572	1	11.572	2.085
	Residual	16.648	3	5.549	
	Total	28.220	4		N.S.
Pioneer sucrose	Regression	254.853	1	254.853	8.473
	Residual	90.231	3	30.077	
	Total	142.191	4		N.S.

fr/gl = summation of fructose and glucose

Table 20.

Changes in sugar content of seeds (mg g^{-1}dry weight seed hr^{-1}) calculated from fitted regression equation for sugar against duration of presoaking treatment of seeds of several crop species (taken over 0 to 96 hours of soaking).

Species	mg g dry weight $seed^{-1}hr^{-1}$		
	Sucrose	Fructose + glucose	Overall
Onward pea	-.2931	+.002910	-.2902
Gladstone pea	-.2639	+.06903	-.1949
Meteor pea	-.2134	+.05488	-.1585
Pioneer pea	-.2103	+.04440	-.1659
maize	-.1699	+.01993	-.1500
broad bean	-.1435	-.03333	-.1768
lettuce	-.1080	-.04725	-.1553
rice	-.05767	+.01956	-.03811
radish	-.05154	+.05599	+.004455
carrot	-.001370	-.1245	-.1259

Figure 7. Change in sucrose content of seeds (mg g^{-1} dry weight seed) with duration of presoaking treatment (0 to 96 hours) in some crop species tolerant of soaking injury.

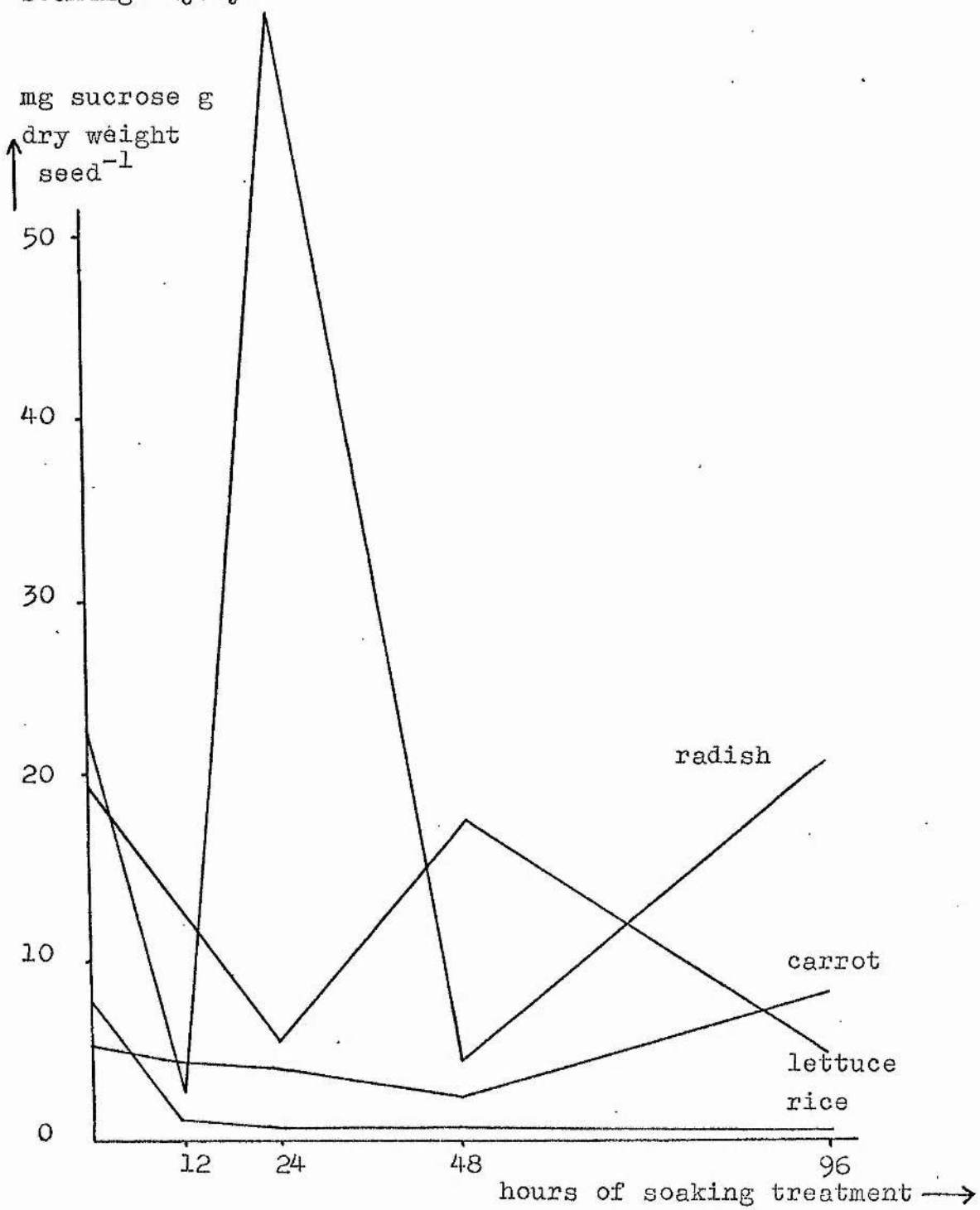
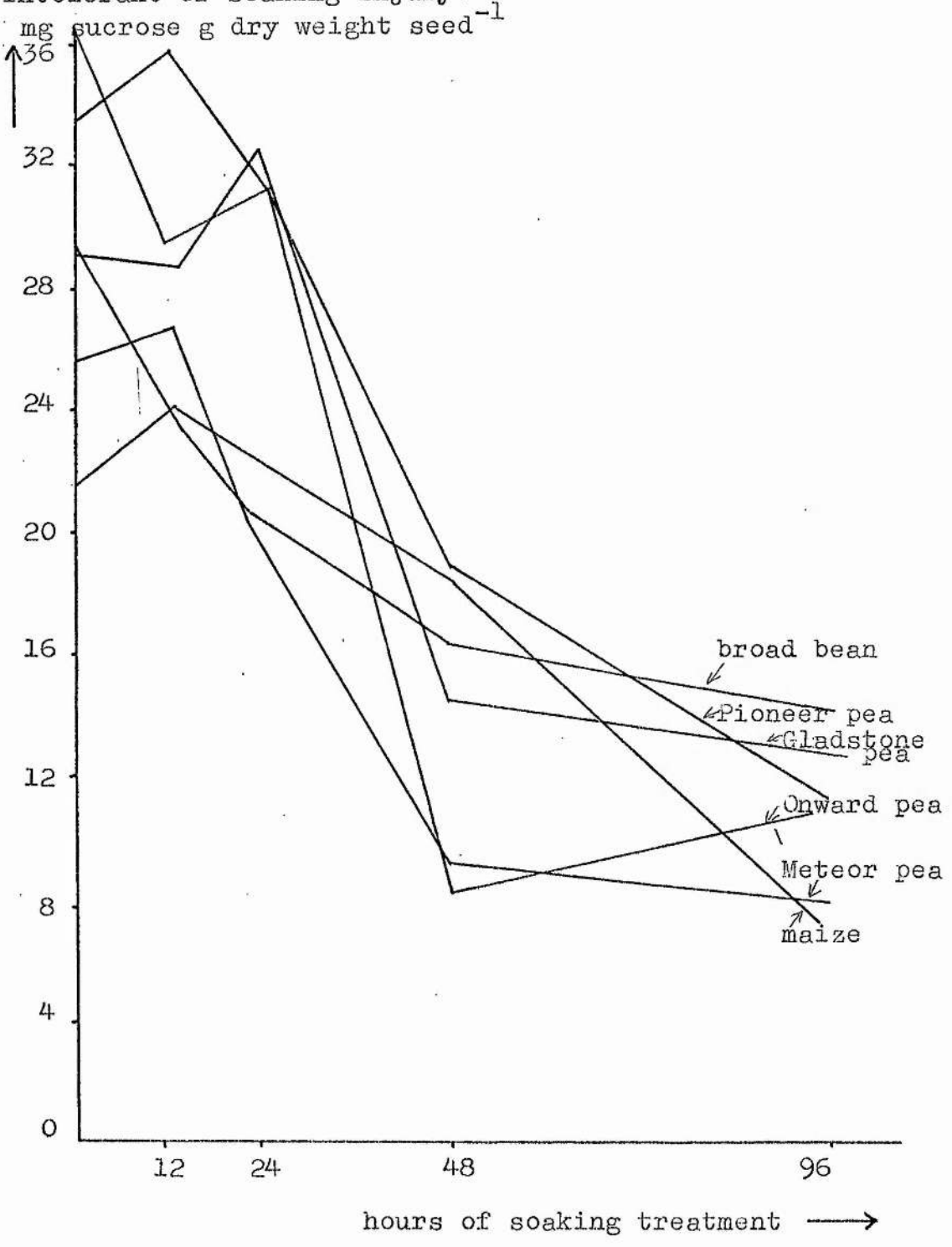


Figure 8. Change in sucrose content of seed (mg g^{-1} dry weight seed) with duration of presoaking treatment (0 to 96 hours) in some crop species which are intolerant of soaking injury.



IV. METABOLISM OF END PRODUCTS OF RESPIRATION

Metabolic variation of genetic origin (eg. soaking tolerance) is most likely to be controlled by enzyme synthesis since the behaviour of a metabolic system depends to a great extent on the presence and properties of enzymes which catalise the reactions involved. Thus, Crawford (1966) showed increased glycolysis on experimental flooding in Senecio and an increase in alcohol dehydrogenase activity in plants intolerant of flooding (1967). He demonstrated an insignificant increase in ADH activity in helophytes while in non helophytes there was up to a seventy fold increase in activity. This led to the suggestion that the accumulation of ethanol as an end product of glycolysis could account for some of the damage caused in flood sensitive species.

Several workers have reported similar ADH induction and alcohol accumulation in seeds. App and Meiss (1958) stated that there is ADH induction in germinating rice seedlings and showed that aeration of the water in which the seeds were germinating reduced ADH activity, but when aeration ceased activity rose again. However, in normal germination, ADH activity decreased by forty percent during the first seven days of germination in pea

seeds (Kolloffel, 1970) and alcohol production largely ceased when the radicle ruptured the seed coat with a reduction in ADH activity (Cossins and Turner, 1962). Anaerobic germination of similar seeds did not show a decline in ADH activity (Kolloffel, 1968; Maffei-Faccioli, 1959).

It is probable therefore, that a control of ADH induction and alcohol accumulation would render seeds more tolerant of soaking. Further, Crawford (1972) reports that there is a diversification of the end products of glycolysis on experimental flooding. For example in those species of bog plants able to withstand flooding, there was an increase in malic acid content of their roots on experimental flooding (Crawford and Tyler, 1969). Also, during temporary anaerobiosis, lactic acid, a non toxic end product accumulates in many tissues. It is therefore likely that the accumulation of non toxic end products of respiration is an additional mechanism of avoidance of soaking injury in seeds.

The presence of lactic acid in seeds under anaerobiosis has been reported by several workers; for example, Sherwin and Simon (1969) showed that the activity of lactic dehydrogenase increased in French bean seeds with dampness of the sand, as it did when the seeds were transferred to a nitrogen

environment, while the accumulation of lactic acid also rose. Wager (1961) and Cossins (1964) also detected lactic acid under anaerobiosis in peas at the start of germination.

A study of the accumulation of alcohol, malic acid and lactic acid will give the relative importance of the accumulation of non toxic end products of respiration.

Changes in the ethanol content of seeds on soaking.

Method - Changes in ethanol on soaking seeds

Seeds of each of ten species were assayed for ethanol after 0, 24, 48 and 96 hours soaking at 25°C. A similar batch were assayed after 96 hours germination on moist filter paper also at 25°C. The seeds were extracted in 6% perchloric acid and deproteinised in an ice bath with 5M K_2CO_3. The supernatant was used in the tests employing a Pye-Unicam spectrophotometer at 340 nm. The assay is detailed in the appendix.

Results

Ethanol was calculated as μM ethanol g^{-1} dry weight seed and is given in Table 21 for species tolerant of soaking injury after soaking from 0 to 96 hours or after germination on moist filter paper. Table 22 summarises the results for species

susceptible to soaking injury. The content of ethanol in all seeds (except lettuce) rose on soaking when compared with controls after 96 hours (Table 23). After soaking increases of .3 to 3.3 times as much ethanol were recorded in soaking tolerant species compared with their controls (96 hours), while 3.8 to 17.0 times as much ethanol was recorded on soaking sensitive species for 96 hours.

The general trend was for an increase in ethanol with increase in duration of soaking, although in some species a distinct fall was recorded at 96 hours, but this is probably due to a decline in metabolism with injury. Regression analyses, however, did not show a significant linear increase of ethanol with duration of soaking treatment (Appendix)*. (This research was concentrating upon changes within the seed and therefore analysis of the soakwater was not carried out. Due to the rapid diffusion rate of ethanol there was possibly some loss into the soakwater from the seed).

The ratio of μM ethanol (g dry weight seed^{-1}) for underwater germination to germination on moist filter paper was calculated for the 96 hour treatments to estimate how much ethanol increased as a result of soaking.

*Tables 69 to 73 Appendix.

Table 21. μ M ethanol g dry weight seed^{-1} after 0, 24, 48 and 96 hours germination underwater and after germination on moist filter paper in seeds of several crop species tolerant of soaking injury.

Species	Hours of treatment				Correlation coefficient for ethanol against hrs of soaking	Ratio 96 hrs under water: 96 air
	0	24	48	96		
rice						
air	.9494	8.4268	14.9850	2.8774	.06951	3.0390
water		6.0538	6.5957	8.7444	.8937	
carrot						
air	20.7846	34.3906	38.6477	38.7312	.7972	2.1444
water		38.6477	145.2421	83.0551	.5556	
lettuce						
air	10.4429	39.9025	22.2267	54.8558	.8071	.3689
water		23.6896	28.5656	20.2357	.4235	
radish						
air	1.6009	4.0725	3.0287	3.5587	.5329	3.3283
water		5.1001	14.8188	11.8442	.7642	

All of the values in the table are means of three replicates.

air= control treatments germinated on moist filter paper.

water= seeds germinated underwater.

Table 22. μM ethanol g dry weight seed^{-1} after 0, 24, 48 and 96 hours germination underwater and after germination on moist filter paper in seeds of several crop species susceptible to soaking injury.

Species	Hours of treatment				Correlation coefficient for ethanol against hrs % of soaking	Ratio 96 hrs under water: 96 air
	0	24	48			
maize						
air	5.1693	34.4186	11.6363	11.7405	-.08423	3.8115
water		10.0869	12.6390	44.7484	.9462	
broad bean						
air	31.1389	23.2300	5.4300	5.1206	-.8826	9.5175
water		6.2490	32.1593	48.7349	.6521	
Meteor pea						
air	10.7387	91.0475	35.8130	9.1305	-.2938	6.6056
water		199.5014	72.0999	60.3127	-.03566	
Onward pea						
air	13.5492	81.7904	47.4158	6.4324	-.3310	16.9588
water		76.4031	127.8380	111.0126	.7753	
Pioneer pea						
air	5.9402	111.6101	86.7794	10.4395	-.1783	9.2999
water		167.9969	175.7670	97.0856	.3117	
Gladstone pea						
air	10.3163	84.3865	48.9366	7.1417	-.2839	6.9804
water		167.9435	131.0726	49.8524	.002494	

All of the values in the table are means of three replicates.
air= control treatments germinated on moist filter paper.
water= seeds germinated underwater.

Table 23. Comparison of the increase in ratio of ethanol (g^{-1}seed) after 96 hours soaking to controls germinated on moist filter paper for 96 hours with the decline in sucrose content of seeds on soaking, and with the rate of respiration under nitrogen in several crop species.

Species	Ratio of ethanol after soaking seeds for 96 hrs: 96 hr germination on filter paper	Anaerobic respiration (QCO_2^n lmg^{-1} dry weight $hour^{-1}$)	Fall in sucrose (mggseed^{-1} hour^{-1})
Onward pea	16.959		-.2931
broad bean	9.518	.269	-.1435
Pioneer pea	9.300	.569	-.2103
Gladstone pea	6.980		-.2639
Meteor pea	6.606	.445	-.2134
maize	3.812	.240	-.1699
radish	3.328	.0616	-.05154
rice	3.039	.103	-.05767
carrot	2.144	.0263	-.001370
lettuce	.3689	.199	-.1080

Changes in malic acid content of seeds on soaking.

Method

Malic acid was estimated in a similar manner to alcohol. The details of the assay are given in the appendix.

Results

Malic acid was calculated as μM malate g dry weight seed^{-1} after 0 to 96 hour soaking treatments and after 96 hours germination on moist filter paper for soaking tolerant species (Table 24) and for soaking intolerant species (Table 25). Regression analyses for malate against duration of soaking treatment are shown in the appendix for each species. Broad bean, and Gladstone pea gave a significant reduction in malate with duration of soaking treatment ($p=.01$) and Meteor and Pioneer peas also gave a significant fall ($p=.05$). (Appendix Tables 74 to 76.)

Table 24. μ M malate g. dry weight seed^{-1} after germination under water for 0 to 96 hours, with controls germinated for 96 hours on moist filter paper, in several crop species tolerant of soaking injury.

Species	Hours of germination underwater				Air	r	96 water
	0	24	48	96	96		:96 air
rice	.6073	1.8442	.3531	.6726	.4877	-.2480	1.379
carrot	29.5492	40.0668	18.9482	42.3205	29.6327	.3303	1.430
lettuce	7.4360	1.1987	5.1605	9.0207	7.8423	.4411	1.150
radish	5.9492	7.9502	3.0936	3.3964	2.5095	-.6775	1.353

All of the values in the body of the table are means of three replicates.

Air = control seeds germinated on moist filter paper in air.

r = correlation coefficient for μ M malic acid against duration of soaking treatment.

Table 25. μM malate g dry weight seed^{-1} after germination under water for 0 to 96 hours, with controls germinated on moist filter paper for 96 hours, in several crop species susceptible to soaking injury.

Species	Hours of germination underwater				Air 96	r	96 water :96 air
	0	24	48	96			
maize	.8290	1.0070	.6944	.3043	.2934	-.8818	1.037
broad bean	2.6888	2.1561	1.9527	1.0191	.8958	-.9926	1.138
Meteor pea	1.1977	1.0298	.4238	.1896	.6866	-.9513	.276
Onward pea	.7746	.4667	.3746	.2997	1.8698	-.8770	.160
Pioneer pea	1.4697	.8537	.6808	.1045	1.4265	-.9716	.073
Gladstone	.3881	.9528	.7762	.0694	3.2526	-.9935	.021

All of the values in the body of the table are means of three replicates.

Air = control seeds germinated on moist filter paper in air.

r = correlation coefficient for μM malic acid against duration of soaking treatment.

Changes in lactic acid content of seeds on soaking.

Method

The extraction and estimation by photometric assay of lactic acid was similar to the method used for alcohol and malic acid. The actual assay used is detailed in the appendix.

Results

Lactic acid was calculated as μ M lactic acid g dry weight seed^{-1} after soaking treatments from 0 to 96 hours and after germination on moist filter paper for 96 hours. The results for several crop species tolerant of soaking injury are shown in Table 26 while those for species susceptible to soaking injury are shown in Table 27.

None of the species gave a significant linear regression for lactic acid against duration of soaking treatment (appendix)(Tables 77 to 79).

A comparison of the relative levels of ethanol, malate and lactate is given in Table 28 where each is expressed as a percentage of the total μM per seed, after 96 hours soaking and in controls (ie. total of ethanol+lactate+malate), together with respiration rates under nitrogen.

Table 26. Changes in the lactic acid content of seeds after soaking for 0 to 96 hours or after germination on moist filter paper for 96 hours in the seeds of several crop species tolerant of soaking injury (μM lactic acid g^{-1} dry weight seed).

Species	Hours of soaking treatment				Air	r	96water
	0	24	48	96	96		:96air
rice	11.036	4.3535	1.5508	1.0090	.1084	-.8461	9.3801
carrot	99.579	283.8063	56.1770	40.6600	40.3172	-.8287	1.0085
lettuce	10.7174	5.1605	36.0423	23.1480	12.6371	-.0175	1.8317
radish	16.175	8.6533	9.6809	5.2891	.8383	-.8908	6.3021

All of the values in the table are the means of three replicates.

air = control seeds germinated on moist filter paper.

r = the correlation coefficient for lactic acid against the duration of soaking treatment.

Table 27. Changes in the lactic acid content of seeds after soaking for 0 to 96 hours and after germination on moist filter paper for 96 hours in seeds of several crop species susceptible of soaking injury (μ M lactic acid g^{-1} dry weight seed).

Species	Hours of soaking treatment				Air	r	96water
	0	24	48	96	96		:96air
maize	3.9011	2.4740	1.0070	1.6146	1.5104	-.4189	1.0690
broad bean	1.5470	1.9229	3.6821	.6850	3.7450	-.5500	.1829
Meteor pea	1.3693	1.3171	1.2910	.6340	.7201	-.9554*	.8809
Pioneer pea	.3693	.1740	2.7774	.5908	.2785	-.0405	2.1214

Each value in the body of the table is a mean of three replicate values.

air = control seeds germinated on moist filter paper in air.

r = the correlation coefficient for lactic acid against duration of soaking treatment.

* = p.05

Table 28. A comparison of ethanol, malic acid and lactic acid as end products of anaerobic respiration after soaking seeds of several crop species for 96 hours underwater or germinated on moist filter paper for 96 hours, when expressed as the total of the three products (μM g^{-1}seed), together with respiration under N_2 for comparison.

Species	% ethanol		% malate		%lactate		QCO_2^n
	water	air	water	air	water	air	
Pioneer pea	99.29	85.96	.11	11.75	.60	2.29	.569
Meteor pea	98.65	86.65	.31	6.52	1.04	6.83	.445
broad bean	96.62	52.46	2.02	9.18	1.36	38.37	.269
maize	95.89	87.03	.65	1.77	3.46	11.20	.240
lettuce	38.61	72.82	17.21	10.41	44.17	16.77	.199
rice	83.87	82.84	6.45	14.04	9.67	3.12	.103
radish	57.69	51.53	16.54	36.34	25.76	12.14	.0616
carrot	50.02	35.64	25.49	27.27	24.49	37.10	.0263

(where % ethanol = $\frac{\mu M \text{ ethanol}}{\mu M \text{ lactate} + \mu M \text{ malate} + \mu M \text{ ethanol}} \times 100$)

QCO_2^n = 1 mg dry weight^{-1} hr^{-1}.

V. CHANGES IN THE PROPERTIES OF ENZYMES AS A RESULT OF SOAKING INJURY IN SEEDS.

In parallel with the study of end products of glycolysis, an investigation of two associated enzymes has been conducted, alcohol and malic dehydrogenases.

McManmon (1968) showed that in non helophytes induction of alcohol dehydrogenase occurred under anaerobic conditions, while there was no such induction in helophytes. When acetaldehyde was added in varying amounts, the enzyme activity varied, such that helophytes required high concentrations of acetaldehyde to produce the maximum reaction velocity, while in non helophytes alcohol dehydrogenase was satisfied at much lower concentrations. He suggested that the differential effect of acetaldehyde contributed towards the acceleration of glycolysis observed on flooding non helophytes. Thus the apparent Michaelis constant of non helophytes fell on flooding, while there was no such fall in helophytes. He then proposed a metabolic scheme to explain the acceleration of glycolysis on flooding non helophytes. This is due largely to the induction of ADH in non helophytes together with malate decarboxylation,

via 'malic enzyme' to pyruvate and then to ethanol. In helophytes, ADH is not induced but a metabolic diversion via PEP carboxylase leads to malate accumulation, 'malic enzyme' being absent.

This section therefore examines the effect of soaking seeds on both ADH and MDH induction and on changes in their apparent Michaelis constants as influenced by acetaldehyde (on ADH) and oxaloacetate (onMDH) concentration.

Changes in alcohol dehydrogenase.

Method - Changes in alcohol dehydrogenase on soaking seeds

Seeds of Pioneer pea, Meteor pea and rice were assayed for ADH activity after 0, 12, 24 and 48 hours submergence in distilled water at 25°C. Each batch was then homogenised in a chilled mortar (except the 0 hour treatment where dry seeds were ground to powder) in Tris-HCl buffer (0.1M, pH 8.0 at 4°C). The homogenate was then passed through two layers of muslin and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was retained for assay.

The spectrophotometric assay was taken from Bergmeyer (1963)(appendix). Protein was estimated by the Lowry method, taking extinction values at 600 nm and comparing with a known albumin graph at the same wavelength.

Results

The specific activity of ADH (mg protein^{-1}) rose on soaking pea seeds up to 48 hours (Table 29) when compared with the dry seed in the two pea cultivars ($p=.01$ and $p=.02$). However, the specific activity of rice ADH fell on soaking when compared with the dry seed (but this was not significant). Specific activities for ADH were much lower in rice than in the two pea cultivars (Figure 9).

When total activity per g seed (μ M g^{-1}dry weight seed) was compared a similar relationship was shown between the three species (Table 30).

Table 29. Change in specific activity of alcohol dehydrogenase (μ M min^{-1} mg $protein^{-1}$) in Pioneer pea, Meteor pea and rice with duration of soaking treatment from 0 to 48 hours.

Treatment hours of soaking	Pioneer pea	Meteor pea	rice
0	.0713	.0463	.0553
S.D.	.005	.005	.021
12	.219	.119	.093
S.D.	.013	.008	.029
24	.175	.140	.059
S.D.	.019	.031	.009
48	.191	.164	.030
S.D.	.020	.025	.006

using a t test for the comparison of 0 and 48 hour treatments the probabilities for chance occurrence of the difference between the means are

Pioneer pea p=.01

Meteor pea p=.02

rice = N.S.

S.D. = standard deviation.

Figure 9. Change in specific activity of ADH with duration of soaking treatment in the seeds of Pioneer pea, Meteor pea and rice (μ M $min^{-1}mg$ $protein^{-1}$ x 1000).

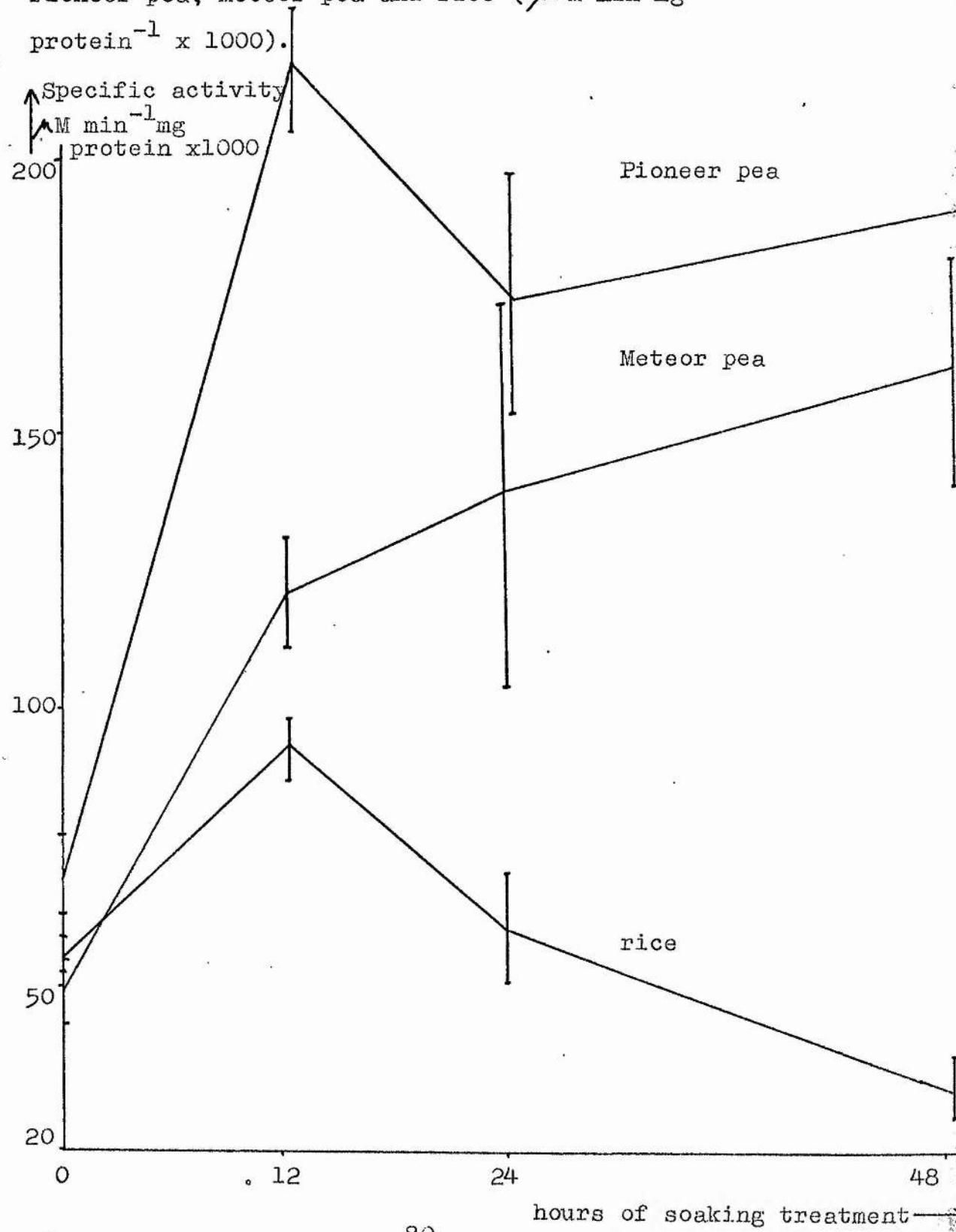


Table 30. Change in total activity of alcohol dehydrogenase per g seed (μ M $min^{-1}g^{-1}$ dry weight seed) in Pioneer pea, Meteor pea and rice with duration of soaking treatment from 0 to 48 hours.

Treatment hours of soaking.	Pioneer pea	Meteor pea	rice
0	15.4350	9.4833	2.7769
S.D.	.9870	1.0945	.3511
12	46.3950	25.8705	2.7226
S.D.	2.5107	2.1202	.7150
24	63.2529	53.5780	1.9566
S.D.	5.9219	13.1993	.3671
48	61.4286	49.2918	2.4673
S.D.	10.2552	2.4455	.1596

t test comparison of 0 and 48 hour means.

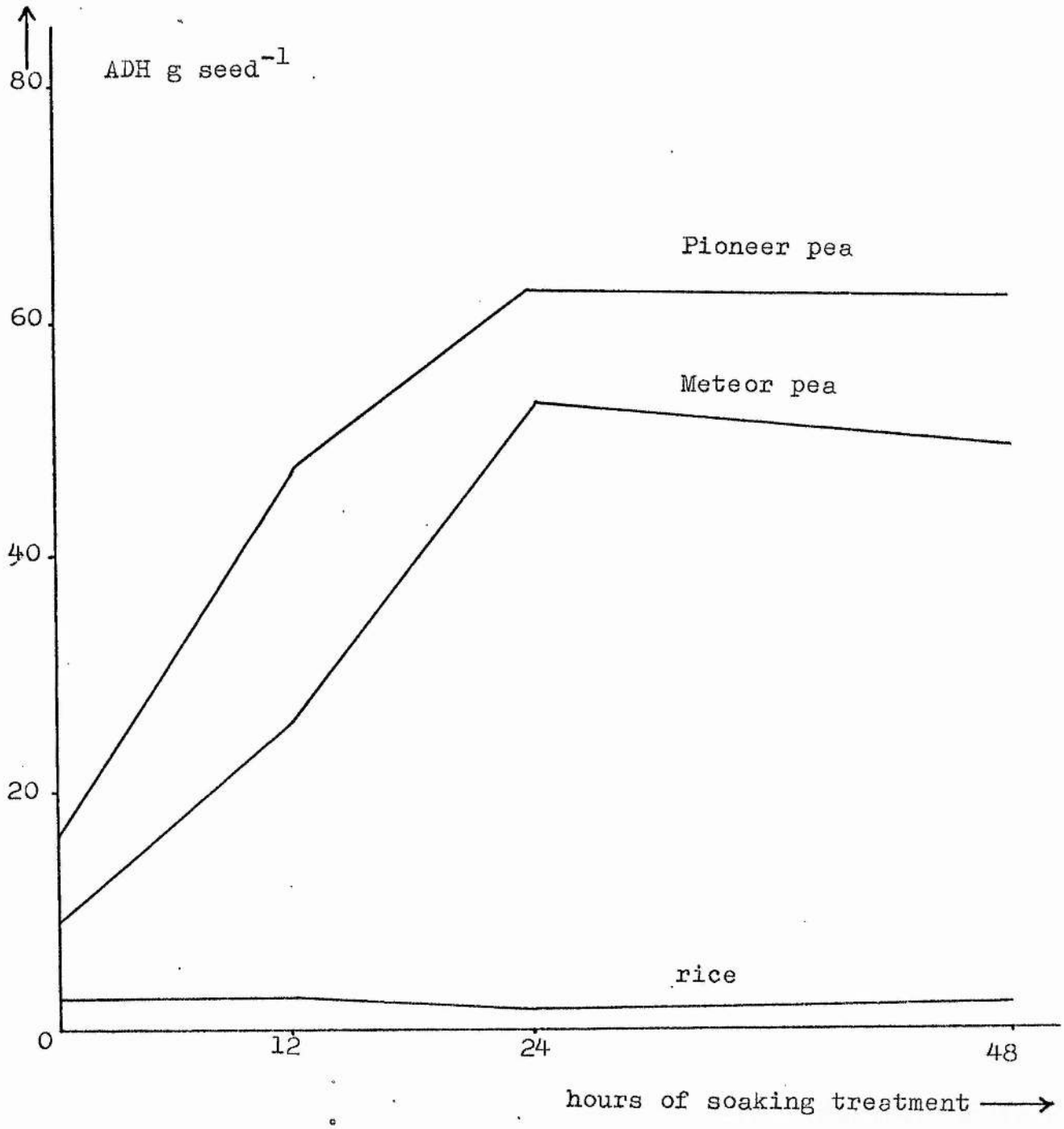
Pioneer pea p = .01

Meteor pea p = .02

rice = N.S.

S.D. = standard deviation.

Figure 10. Change in total activity of alcohol dehydrogenase per g seed (μ M min^{-1} g dry weight $seed^{-1}$) in Pioneer pea, Meteor pea and rice with duration of soaking treatment from 0 to 48 hours.



Changes in Michaelis constants of ADH on soaking seeds of Pioneer pea, Meteor pea and rice.

Method

The Michaelis constants for ADH were determined by plotting the reciprocal of the reaction velocity against the reciprocal of the substrate concentration when different substrate concentrations were used. The straight line relationship cuts the base line when extrapolated at -1/Km (the reciprocal of the Michaelis constant)(Figure 11).

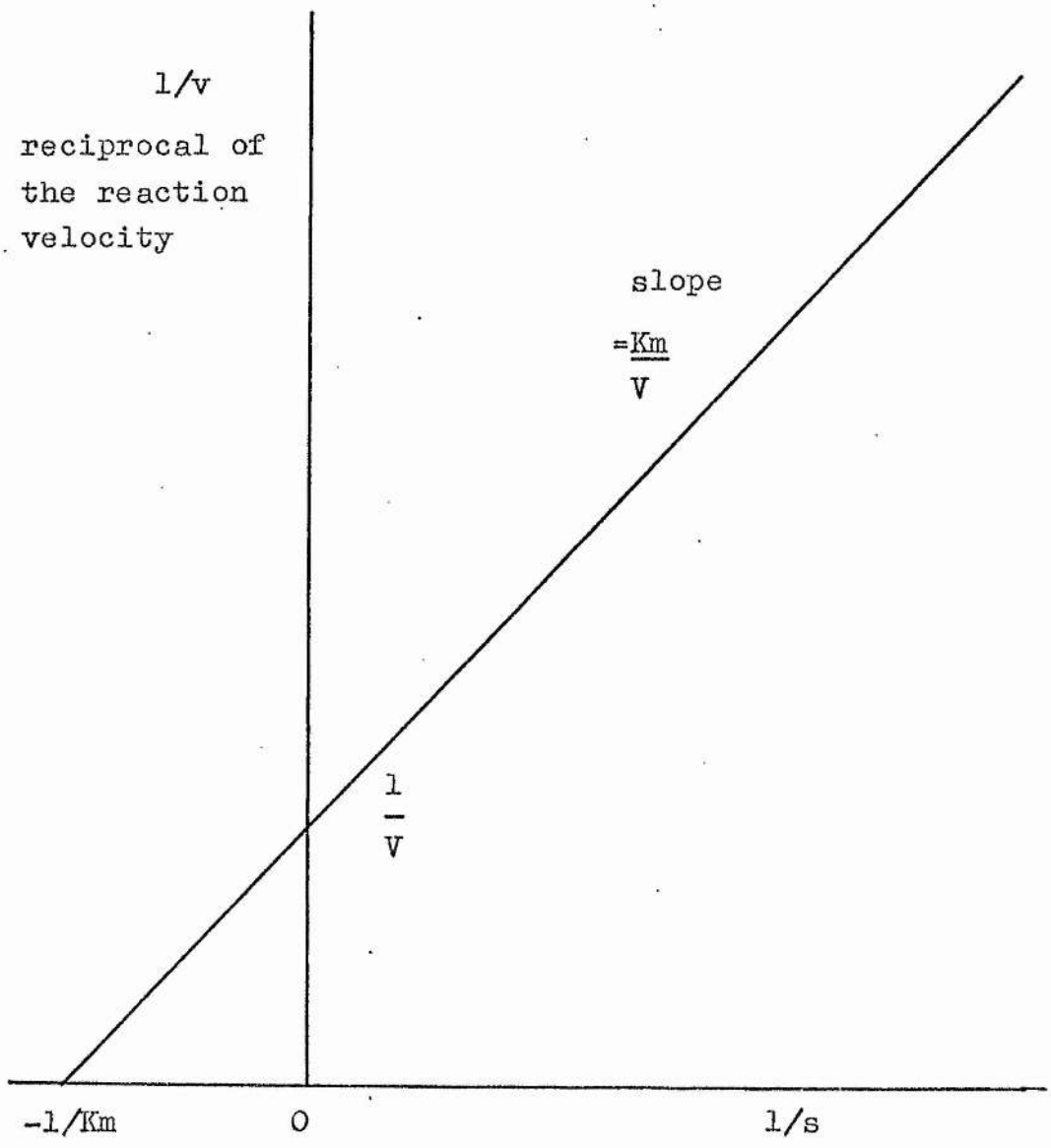
Results

Table 31 gives the mean Km values ($M \times 10^{-3}$) for ADH with respect to acetaldehyde using extracts from Pioneer pea, Meteor pea and rice, with standard deviations based on three replicates. (The reaction velocities were plotted as change in $\mu M\ min^{-1} mg\ protein^{-1}$).

No clear trend for Km with duration of soaking was established, but the lowest Michaelis constants were recorded in the least tolerant species (Pioneer pea) and the highest in the more tolerant species (rice)(Figure 12).

A summary of the changes of alcohol and alcohol dehydrogenase is given in Table 32.

Figure 11. Method for the determination of Michaelis constants using the Lineweaver-Burk plot



v = reaction velocity

V = maximum reaction velocity

s = substrate concentration

Km = Michaelis constant

Table 31. Changes in the Michaelis constant for ADH in seeds of Pioneer pea, Meteor pea and rice after soaking for varying lengths of time (0 to 48 hours)(M x 10^{-3} acetaldehyde).

Treatment hours of soaking.	Pioneer pea	Meteor pea	rice
0	.132	.315	.303
S.D.	.0107	.128	.0364
12	.442	.381	1.129
S.D.	.0906	.0403	.345
24	.180	.222	.430
S.D.	.0273	.0244	.0656
48	.302	.412	.469
S.D.	.0721	.0338	.140

Each figure is the mean of three replicates where S.D.= standard deviation.

Figure 12. Changes in the Michaelis constant of ADH with length of soaking treatment in Pioneer pea, Meteor pea and rice (M x 10^{-3} acetaldehyde).

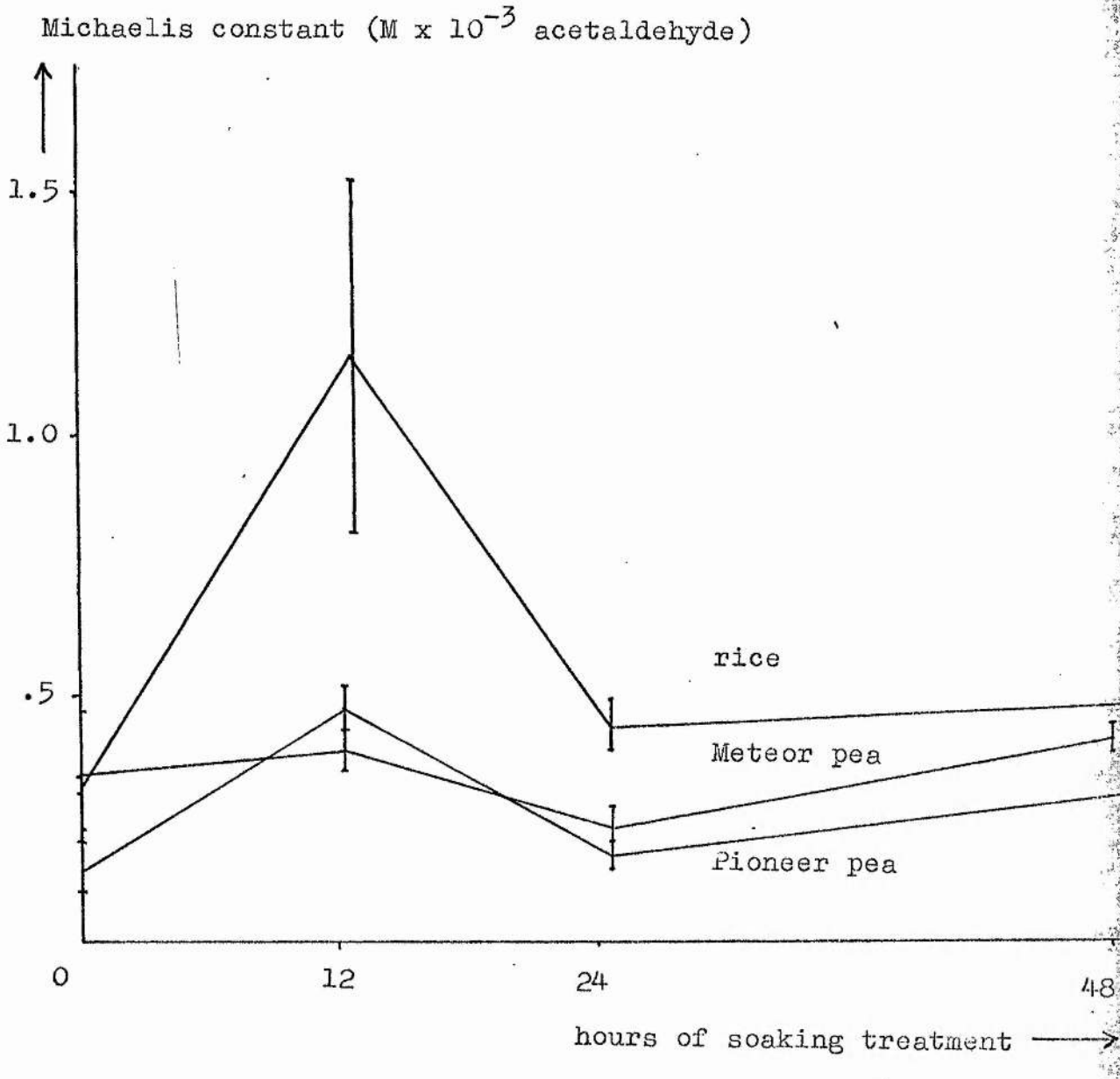


Table 32. Summary of changes in alcohol, alcohol dehydrogenase and its Michaelis constant on soaking seeds of Pioneer pea, Meteor pea and rice.

	Species		
	Pioneer pea	Meteor pea	rice
1.Alcohol (μMg^{-1} dry weight seed)			
96 hours soaking	97.0856	60.3127	8.7444
48 hours soaking	175.7670	72.0999	6.5957
2.Specific activity ADH ($\mu M\ min^{-1} mg^{-1}$ protein)			
48 hours soaking	.191	.164	.0300
3.Activity of ADH ($\mu M min^{-1}$ g dry wt seed^{-1})			
48 hours soaking	61.423	49.292	2.467
4.Michaelis constant ADH with respect to acetaldehyde($Mx10^{-3}$)			
48 hours soaking	.302	.412	.469

Changes in malic dehydrogenase on soaking seeds.

Method

The experiments were conducted in a similar manner to alcohol dehydrogenase, with the exception of the assay (appendix).

Results

Table 33 gives the mean specific activities of MDH (μM min^{-1} mg $protein^{-1}$) in relation to the length of soaking treatment (0 to 48 hours) in Pioneer pea, Meteor pea and rice. Standard deviations based on three separate plant extracts are also given.

The total activity per g dry weight of seed of MDH did not show any clear trend with duration of soaking treatment, nor was there any difference in the relative levels of MDH in the three species after 48 hours of soaking (Table 34).

When specific activity and activity per seed at 0 hours were compared with 48 hour soaking treatments, a significant rise on soaking was given in Pioneer pea ($p=.05$), in Meteor pea ($p=.02$) but in rice there was no significant change (Tables 33 and 34). Figures 13 and 14 show changes in specific activity and activity per g seed in graph form.

Table 33. Change in specific activity of malic dehydrogenase ($\mu M\ min^{-1}\ mg\ protein^{-1}$) with duration of soaking in Pioneer pea, Meteor pea and rice seeds.

Hours of soaking	Species		
	Pioneer pea	Meteor pea	rice
0	.170	.112	.478
S.D.	.002	.008	.175
12	.234	.329	.745
S.D.	.066	.071	.055
24	.267	.193	.728
S.D.	.023	.011	.177
48	.220	.270	.248
S.D.	.046	.035	.060

Each figure is the mean of three replicates where S.D. = standard deviation.

t test for comparison of 0 and 48 hour means.

Pioneer pea	$p = .025$
Meteor pea	$p = .02$
rice	N.S.

Table 34. Change in activity of malic dehydrogenase per g seed (μM min^{-1}g dry weight $seed^{-1}$) in Pioneer pea, Meteor pea and rice with duration of soaking treatment (0 to 48 hours).

Hours of soaking	Species: Pioneer pea	Meteor pea	rice
0	36.8243	22.8013	24.1621
S.D.	.3998	1.6265	6.4475
12	51.6289	71.5649	24.6728
S.D.	16.2635	14.5579	.4469
24	97.2567	73.4019	23.6195
S.D.	11.1701	5.4535	5.2027
48	67.9104	81.8710	19.7893
S.D.	11.3430	6.8809	2.0109

Each figure is the mean of three replicates where S.D.= standard deviation.

t test for the comparison of 0 and 48 hour means.

Pioneer pea	p = .05
Meteor pea	p = .01
rice	N.S.

Figure 13. Changes in the specific activity of MDH (M x 1000)(μM min^{-1} $mg\ protein^{-1}$) with duration of soaking treatment in Pioneer pea, Meteor pea and rice.

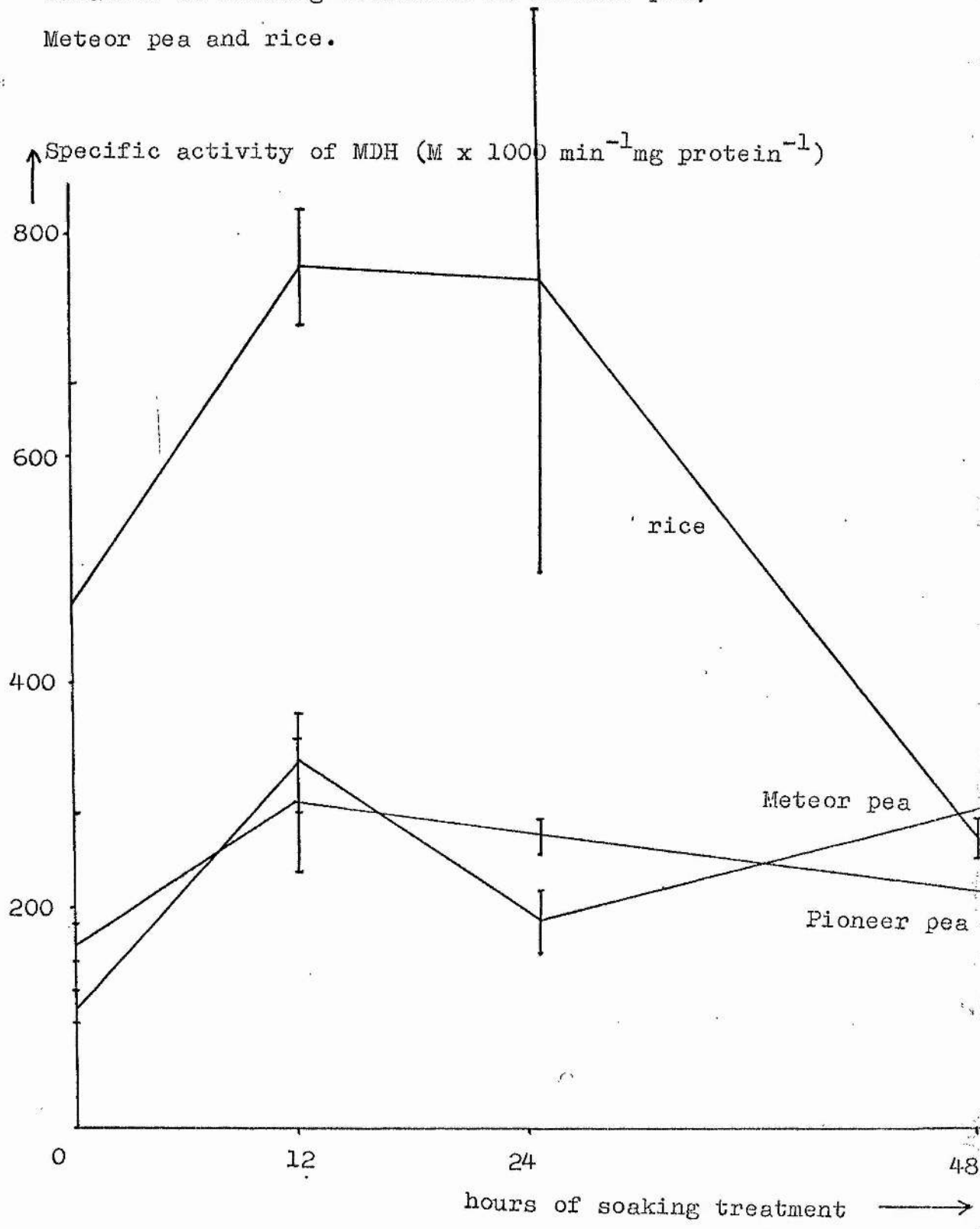
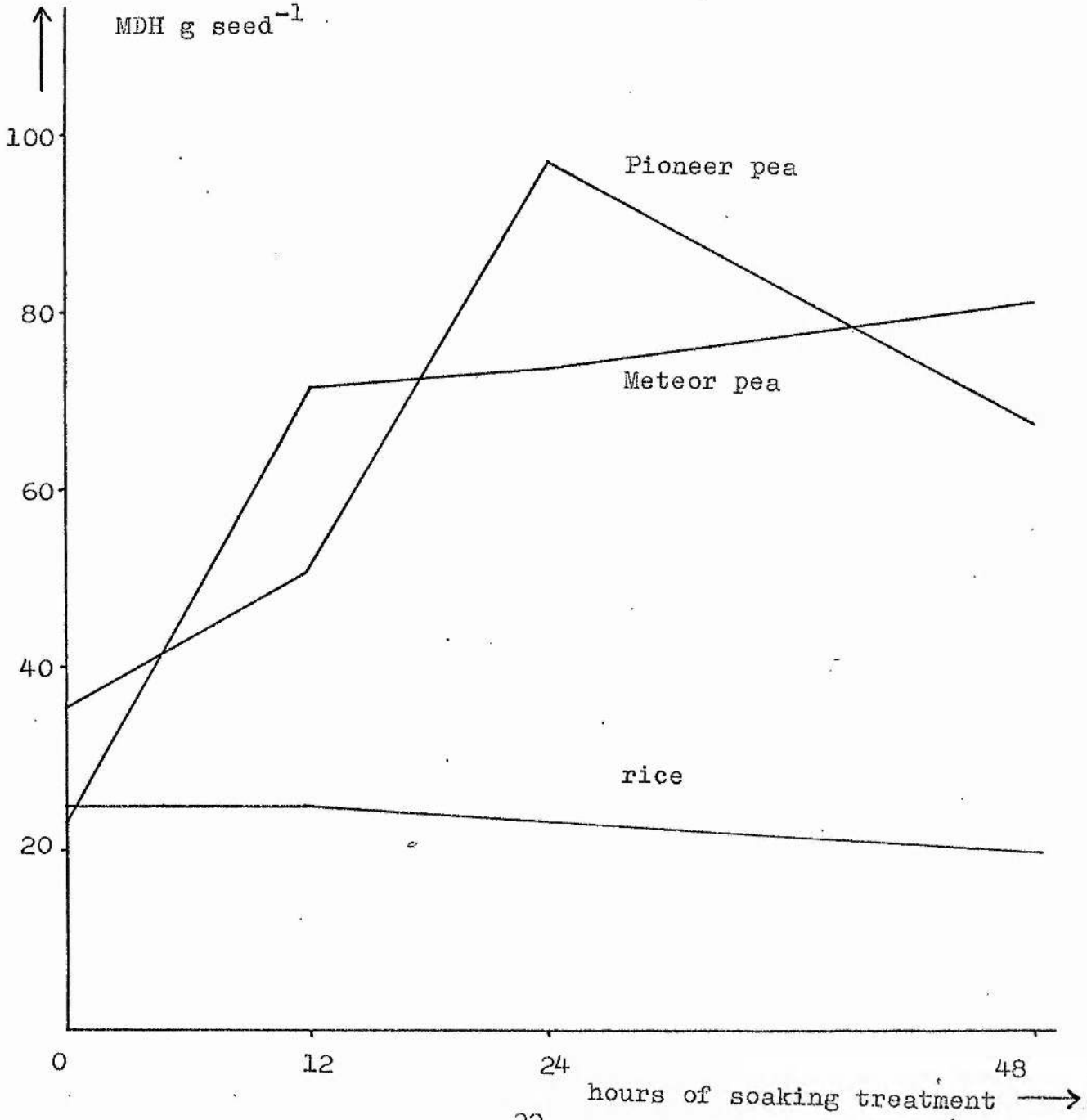


Figure 14. Change in activity of Malic Dehydrogenase per g seed (μ M min^{-1} g dry weight $seed^{-1}$) in Pioneer pea, Meteor pea and rice with duration of soaking treatment (0 to 48 hours).



Changes in the Michaelis constants of malic dehydrogenase on soaking seeds of Pioneer pea, Meteor pea and rice.

Method

The Michaelis constants for MDH were determined in the same way as for ADH but using oxaloacetate as the substrate.

Results

Table 35 gives the changes in Km values on soaking from 0 to 48 hours as the means of three replicates with standard deviations. The Michaelis constant showed no change in the two pea cultivars, but it rose in rice on soaking although this failed to reach significance. However, after 48 hours soaking the actual Km for rice was lower than either of the pea cultivars (Figure 15).

A summary of changes in malic acid, malic dehydrogenase and its Michaelis constant on soaking seeds is given in Table 36. The lowest values for malic acid and MDH were recorded in Pioneer pea, intermediate values for Meteor pea and the highest values in rice. The Michaelis constants for MDH showed the reverse trend.

Table 35. Changes in Michaelis constants for malic dehydrogenase (M x 10^{-3} oxaloacetate) with duration of soaking treatment in seeds of Pioneer pea, Meteor pea and rice.

Hours of soaking	Species		
	Pioneer pea	Meteor pea	rice
0	.345	.190	.0895
S.D.	.0069	.0323	.0180
12	.0613	.0788	.0817
S.D.	.0062	.0116	.0090
24	.250	.150	.210
S.D.	.0479	.0250	.0247
48	.224	.199	.112
S.D.	.0903	.0285	.0089

Each figure in the table is the mean of three replicates where S.D.= standard deviation.

t test for the comparison of 0 and 48 hour means.

Pioneer pea N.S.

Meteor pea N.S.

rice N.S.

Figure 15. Changes in Michaelis constants for malic dehydrogenase (M x 10^{-3}) with duration of soaking treatment in Pioneer pea, Meteor pea and rice.

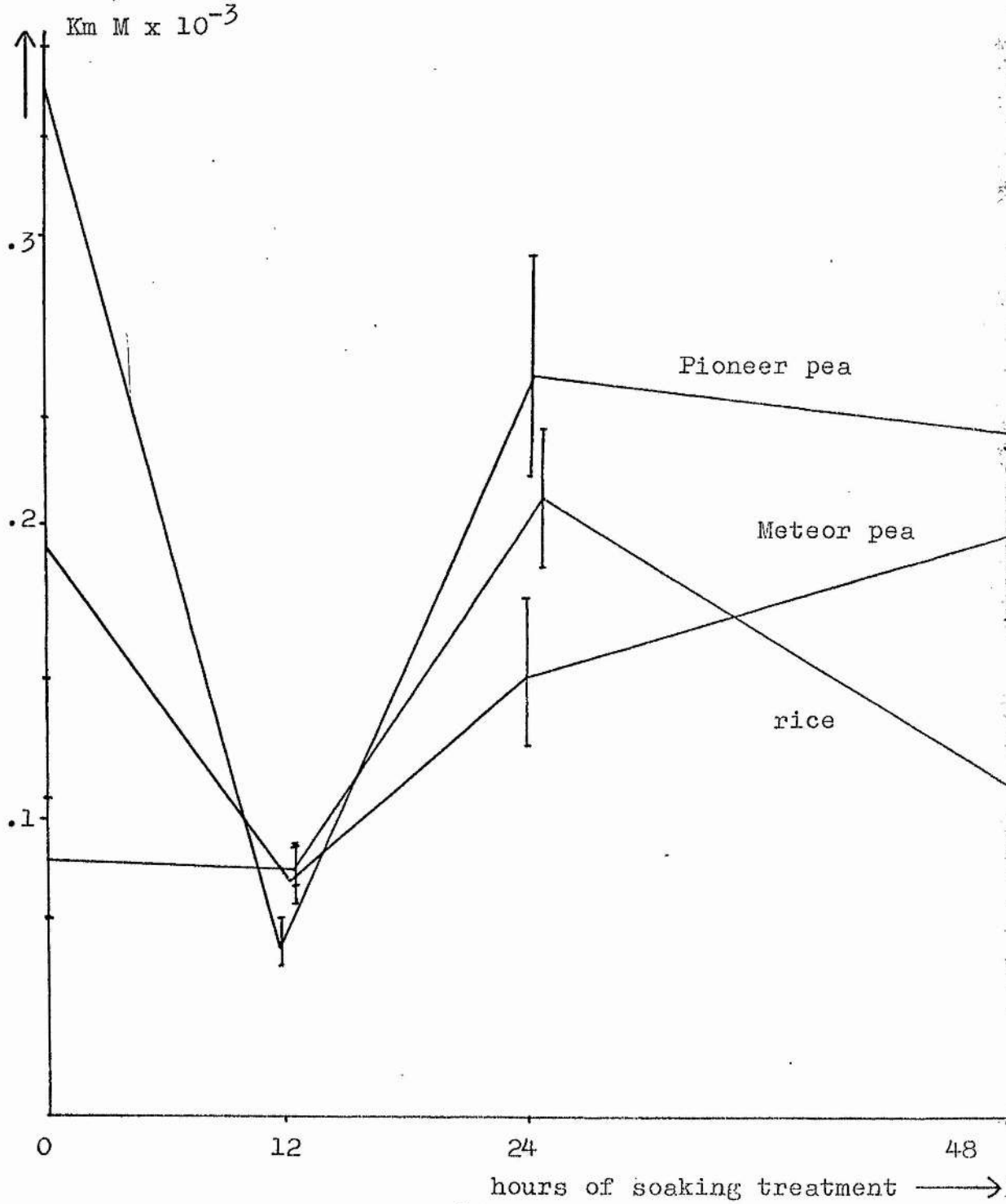


Table 36. Summary of changes in malic acid, malic dehydrogenase and its Michaelis constant on soaking seeds of Pioneer pea, Meteor pea and rice.

	Species		
	Pioneer pea	Meteor pea	rice
1.Malic acid (μMg dry wt seed^{-1})			
96 hours water	.1045	.1896	.6726
48 hours water	.6808	.4328	.3531
2.Specific activity of MDH(μM min^{-1}mg protein^{-1})			
48 hours water	.220	.270	.248
3.Activity g seed^{-1} (μM min^{-1}g dry wt seed^{-1})			
48 hours water	67.910	81.871	19.789
4.Michaelis constant MDH (Mx10^{-3}oxaloacetate)			
48 hours water	.224	.199	.112

B. THE EFFECT OF CHANGE IN TEMPERATURE ON SOAKING INJURY.

Flooding injury in plants is largely controlled by a regulation of glycolysis (Crawford, 1972), and therefore not only the direction but also the rate of the reactions involved in glycolysis will be critical in determining the extent of injury. If temperature is increased then the rate of glycolysis will rise (because of the effect of temperature on metabolic reactions), and will probably result in greater damage to the seeds on soaking.

However, there is normally a critical temperature below which respiration will become slower, and there is much evidence for low temperature damage to seeds. For example, emergence failures as a result of low temperature imbibition have been reported in cotton (Christiansen, 1968 and 1969) and in lima beans (Pollock and Toole, 1966; Pollock, 1969) while chilling during germination reduced survival in crimson clover (Hoveland and Elkins, 1965), in cacao (Ibaney, 1963), in peas (Highkin and Lang, 1966) and in beans (Pollock, Roos and Manalo, 1969).

This section examines temperature effects on soaking tolerance in seeds both by germination

and by metabolic studies in order to determine both the presence and nature of low temperature damage and damage resulting from higher temperatures during soaking.

I. GERMINATION EXPERIMENTS

Underwater germination of seeds in relation to temperature.

Method

Thirty seeds of each of ten crop species were placed in beakers full of distilled water (50 ml beakers for the smaller seeded species and 250 ml beakers for the larger seeded species) in controlled temperature incubators in darkness. Replicate batches were placed in incubators at each of four temperatures (0, 10, 20 and 30°C) for seven days. After seven days the number of seeds in which radicle emergence had taken place was recorded.

Results

The effect of temperature on under water germination of seeds of ten crop species is shown as the percentage of 30 seeds showing radicle emergence at seven days (in each of four temperature treatments) in Table 37 and Figure 16. Probabilities for the difference between temperature treatments within each species is given in Table 38.

Table 37. The effect of temperature on under water germination in seeds of several crop species after seven days submergence (percentage germination where germination is taken as the number of seeds out of 30 with radicle emergence).

Species or Cultivar	Temperature of soaking 0	10	20	30°C
rice	0	83.3	100	96.7%
carrot	0	63.3	93.3	50.0%
lettuce	0	90.0	76.7	33.3%
radish	0	70.0	96.7	50.0%
maize	0	3.3	6.7	0 %
Meteor pea	0	0	0	0 %
Pioneer pea	0	0	0	0 %
Onward pea	0	0	0	0 %
Gladstone pea	0	0	0	0 %
broad bean	0	0	0	0 %

Figure 16. The effect of temperature on under water germination of species tolerant of soaking injury.

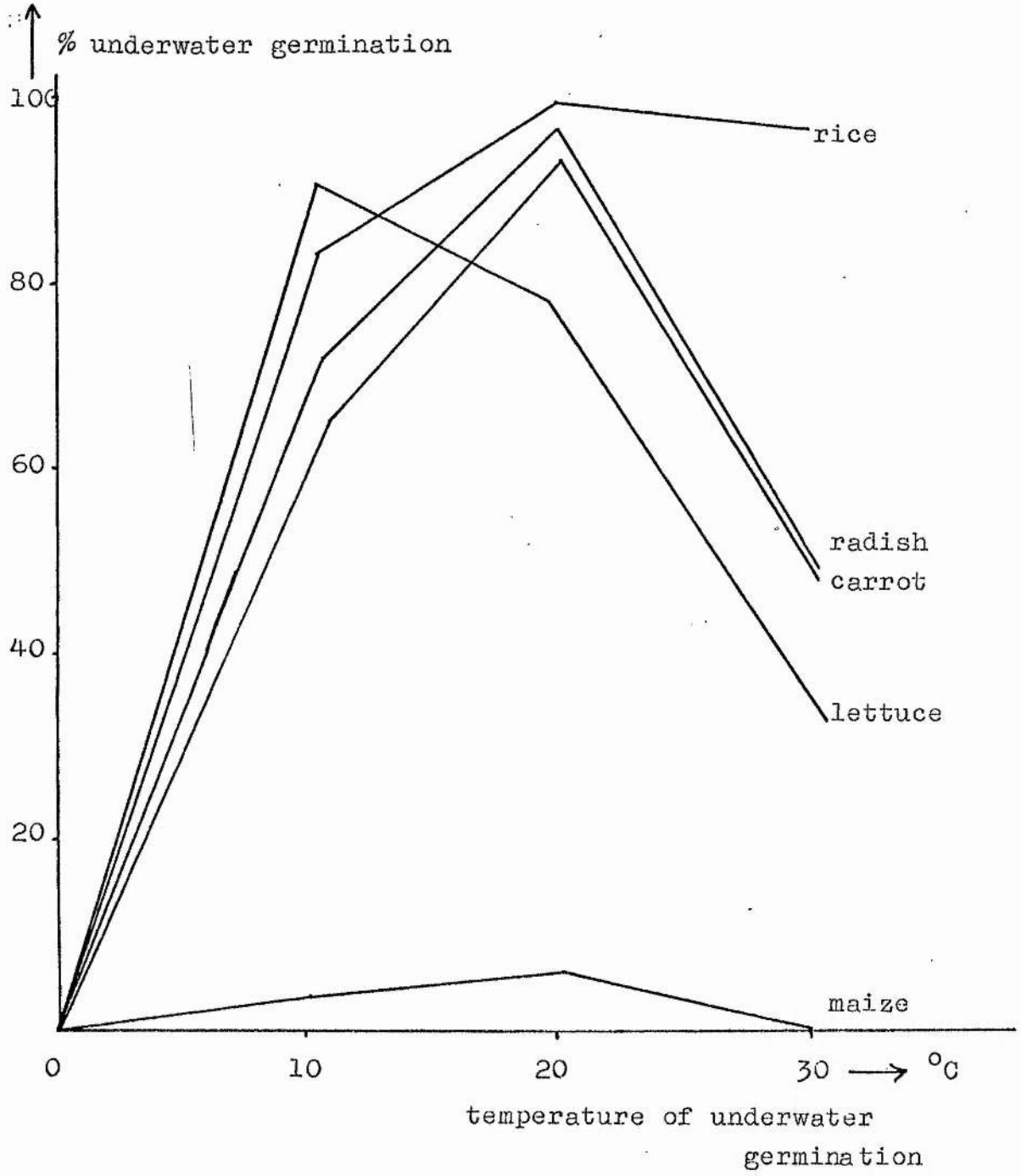


Table 38.

Probability tests for the difference between under water germination at different temperatures within each of several crop species.

Species or cultivar	Temperatures between which comparisons are made, with probabilities for their difference due to chance occurrence.		
	0 + 10°C	10 + 20°C	20 + 30°C
rice	2.75×10^{-12}	.0261	.500
carrot	2.70×10^{-8}	.0051	.0018
lettuce	4.61×10^{-14}	.149	.000107
radish	1.79×10^{-9}	.0061	.000031
maize	.500	.310	.250
Meteor, Onward, Pioneer, Gladstone peas.	1.0	1.0	1.0
broad bean	1.0	1.0	1.0

Temperature of seed presoaking treatments in relation to subsequent germination and development.

Method

Thirty seeds of each of ten crop species were soaked in distilled water for 0, 24, 48 and 96 hours at 0, 10, 20 and 30°C in all combinations in incubators in darkness. All of the treatments were timed so that all of the seeds were sown at the same time in trays of sand in the greenhouse at about 25°C. A fourteen day germination count was taken (where germination was taken as the number of seedlings emerging above the sand).

Results

The number of seedlings emerging above the germination medium at 14 days is given in Tables 39 (soaking tolerant species) and 40 (soaking intolerant species) after presoaking treatments of 0, 24, 48 and 96 hours at 0, 10, 20 and 30°C in all combinations in ten crop species.

The species previously recorded as sensitive to soaking injury showed a marked fall in germination after presoaking at 0 and at 30°C when compared with 20 and 30°C treatments. However, the species tolerant of soaking injury were less sensitive to an increase in soaking injury at 0 and 30°C. These results are represented in Figures 17 to 26

in diagrammatic form. Figures 27 and 28 show the overall effect of low ($0^{\circ}C$) and high ($30^{\circ}C$) temperature soaking treatments on subsequent germination.

Table 39. Number of seedlings emerging above the germination medium after presowing soaking treatments from 0 to 96 hours at 0 to 30°C in soaking tolerant species.

Species/ cultivar	Duration presoak hours	Temperature of presoaking 0	10	20	30°C
rice	none	24			
	24	22	23	23	29
	48	12	23	23	29
	96	24	22	27	21
carrot	none	30			
	24	21	23	20	24
	48	9	23	17	23
	96	18	20	25	15
lettuce	none	26			
	24	26	24	30	28
	48	25	26	27	23
	96	28	27	26	26
radish	none	25			
	24	29	26	28	29
	48	25	28	27	17
	96	25	27	26	14

(number of seeds germinating out of 30).

Table 40. Number of seedlings emerging above the germination medium after presowing soaking treatments from 0 to 96 hours at 0 to 30°C in soaking sensitive species (the values for the pea cultivars are means of two replicates).

Species/ cultivar	Duration soak hrs.	Temperature of presoaking 0	10	20	30°C
Meteor pea	none	25			
	24	3	21.5	22.5	12.5
	48	3	18	23.5	0
	96	1	7	14.5	0
Pioneer	none	19			
pea	24	2	7.5	12	8
	48	0.5	6	10	0
	96	5	12	2	0
Onward	none	27			
pea	24	5.5	14.5	17	9.5
	48	1	13	8.5	0
	96	0	17.5	3.5	0
Gladstone	none	22			
pea	24	6.5	20	14.5	14
	48	5	17	19.5	0
	96	0	0	1	0
broad bean	none	29			
	24	7	13	4	12
	48	10	14	1	1
	96	6	3	2	0
maize	none	29			
	24	23	20	25	20
	48	6	16	24	19
	96	11	16	25	11

(number of seeds germinating out of 30).

Figures 17 - 20. Germination of seeds of several crop species after presoaking at different temperatures for varying lengths of time.

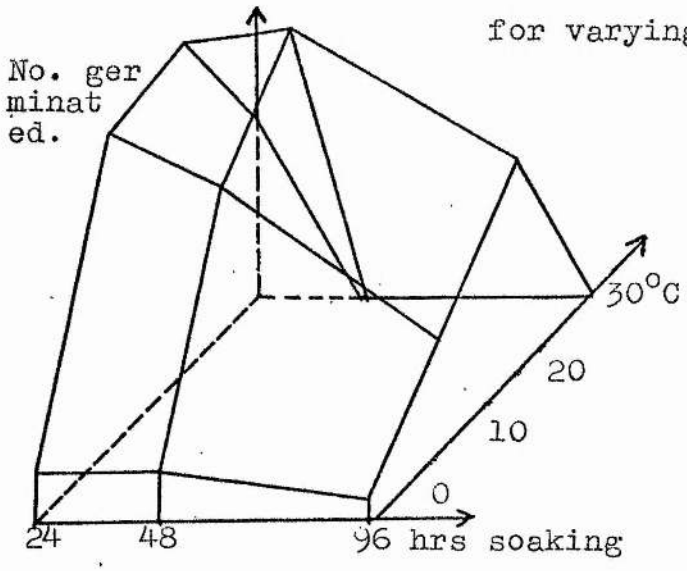


Figure 17. Meteor pea

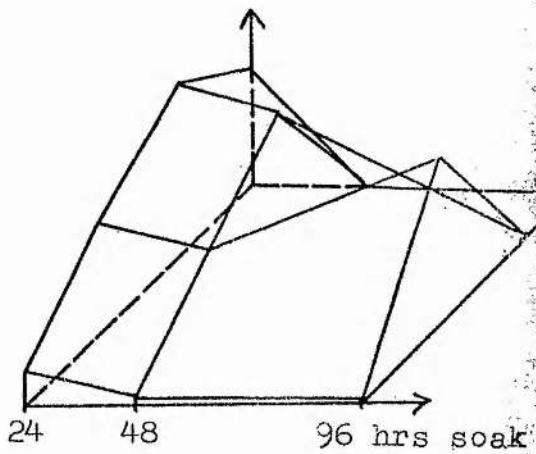


Figure 18. Pioneer pea

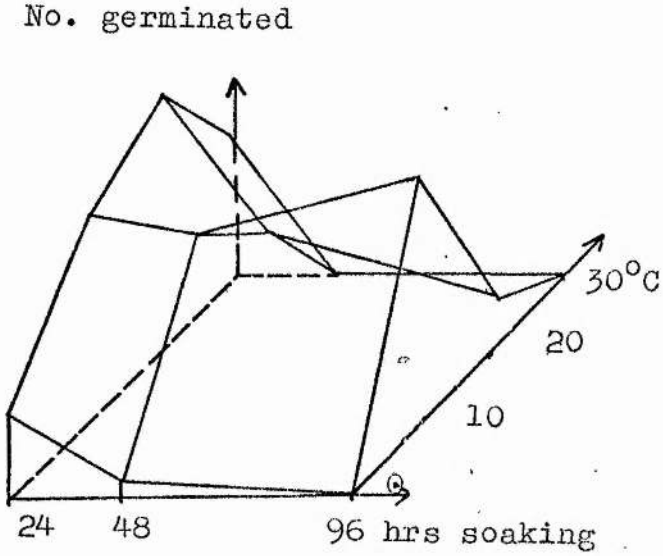


Figure 19. Onward pea

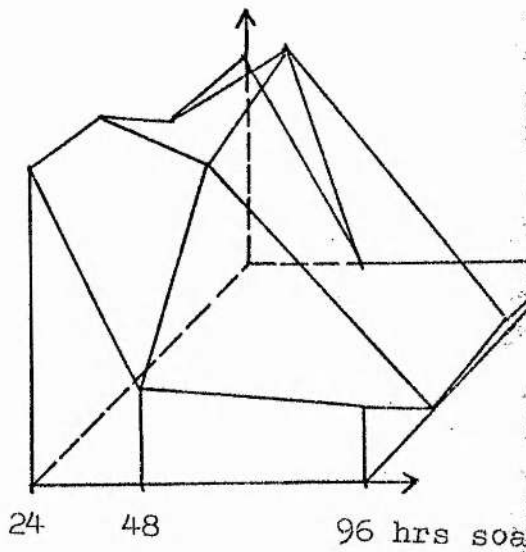


Figure 20. Gladstone pea

Figures 21 and 22. Germination of seeds of two crop species after presoaking at different temperatures for varying lengths of time.

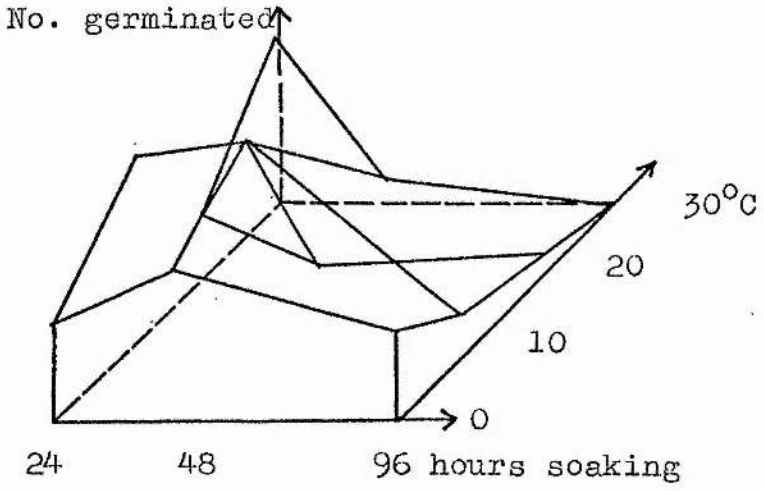


Figure 21. Broad bean

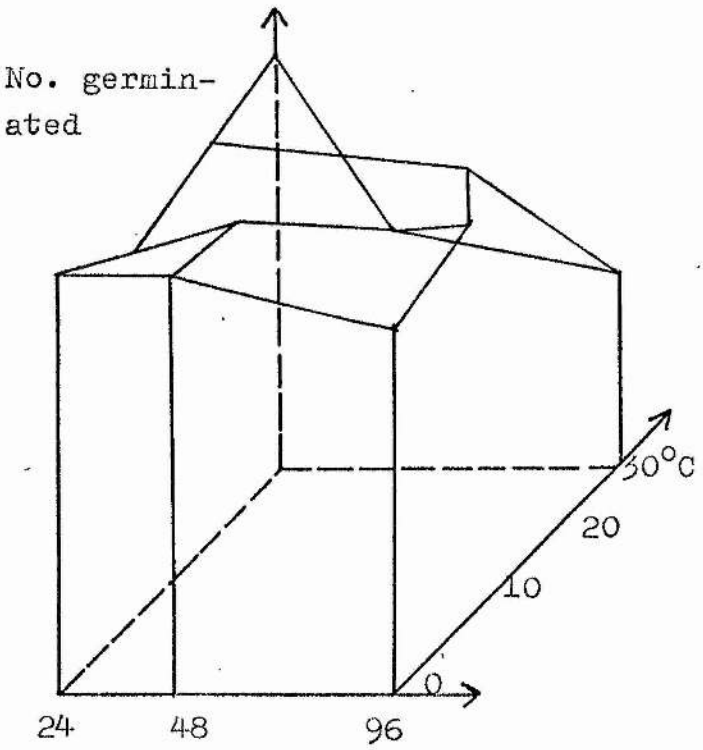


Figure 22. Radish

Figures 23 to 26. Germination of seeds after presoaking at different temperatures for different lenths of time.

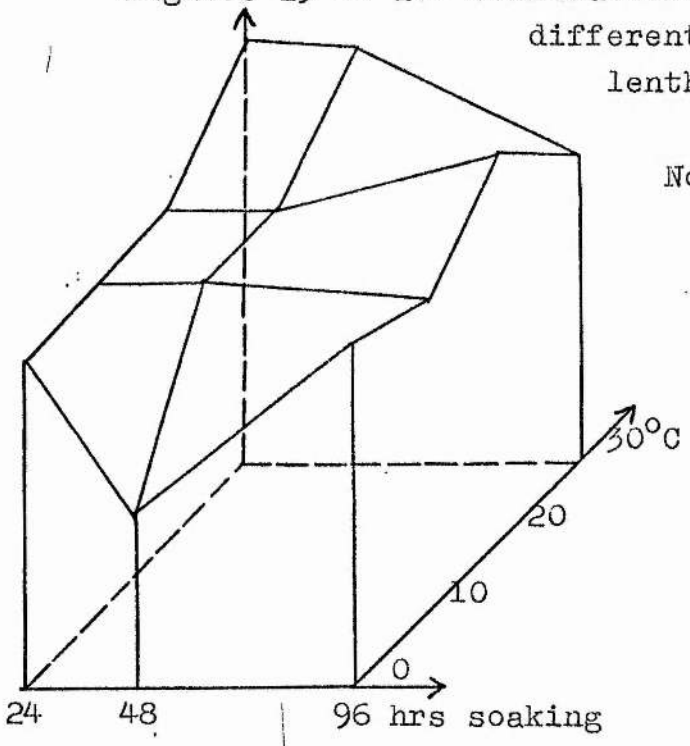


Figure 23. Rice

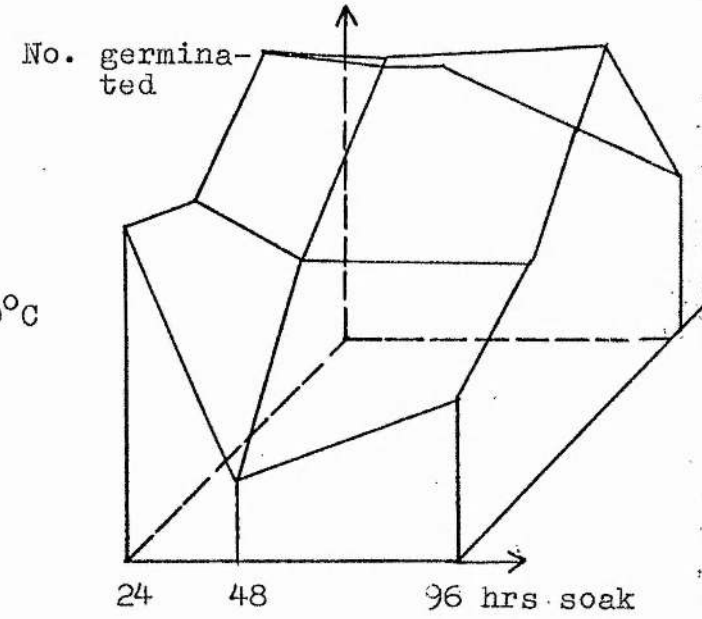


Figure 24. Maize

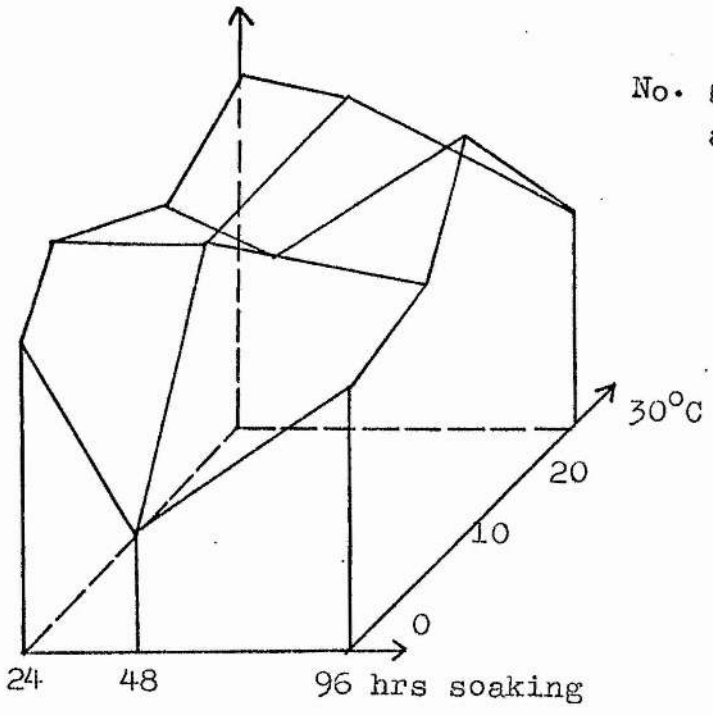


Figure 25. Carrot

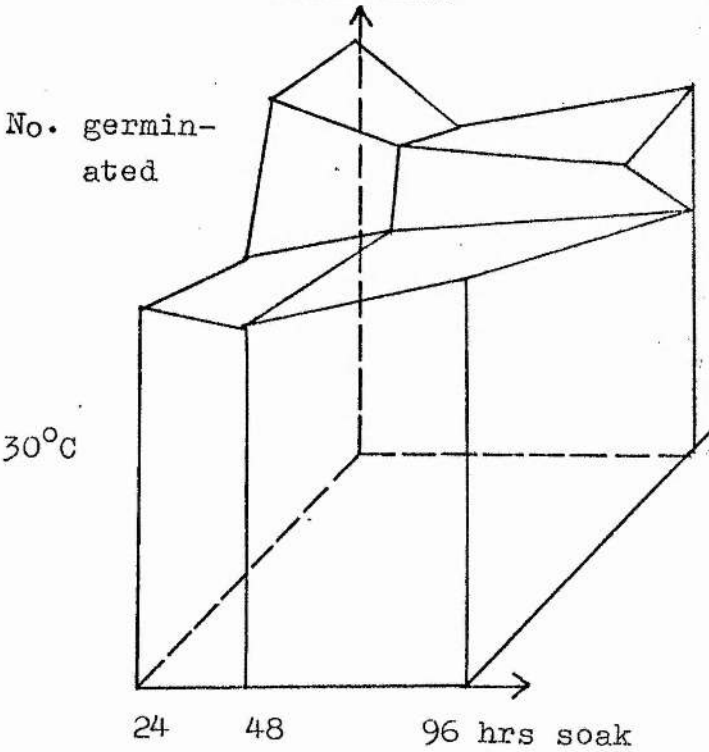


Figure 26. Lettuce

Figure 27. The effect of duration of presowing soaking treatment at 0°C on subsequent germination at 20-25°C.

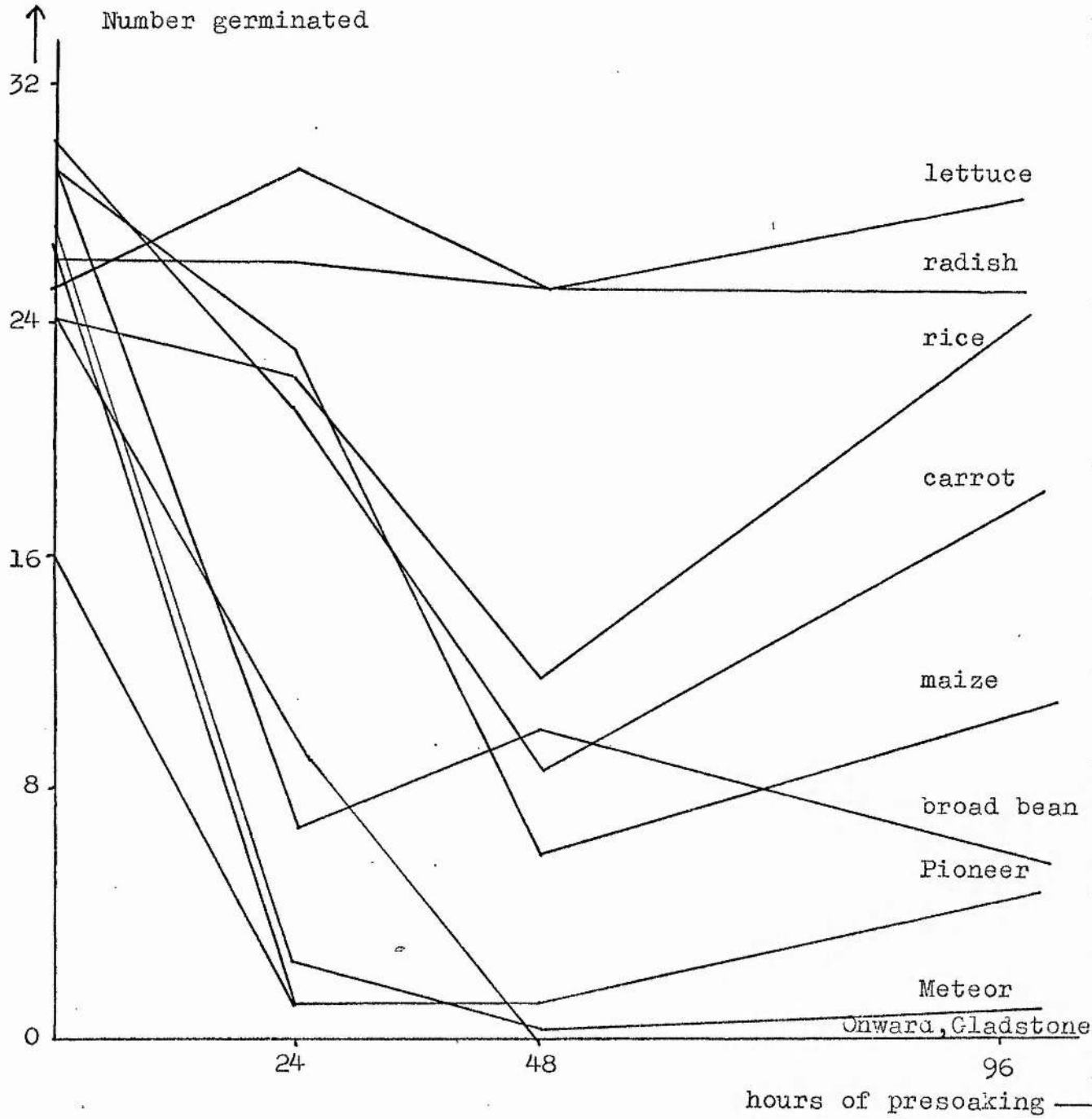
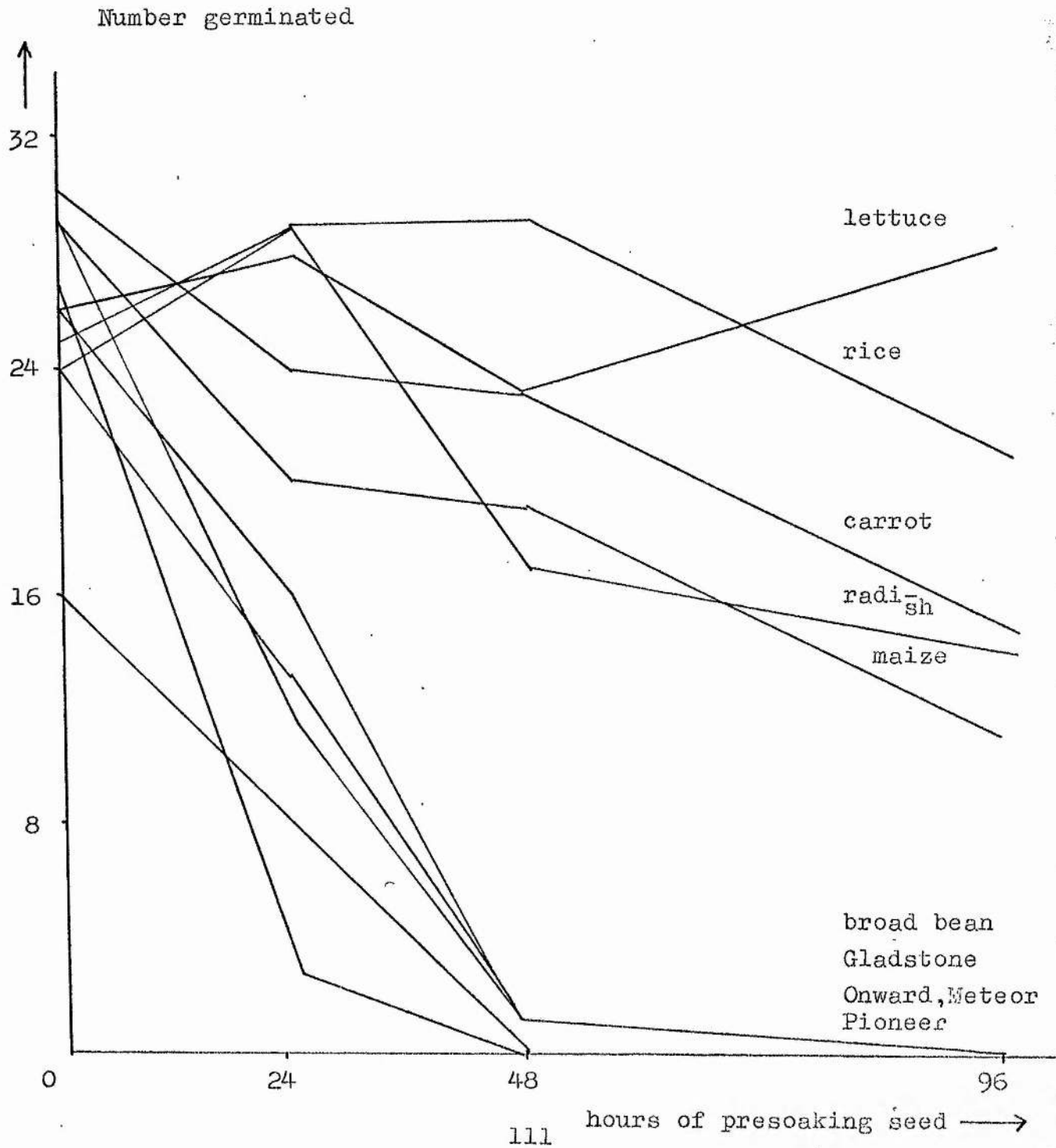


Figure 28. The effect of presowing soaking treatment at 30°C on subsequent germination at 20-25°C.



II. CHANGE IN THE RATE OF RESPIRATION UNDER NITROGEN WITH TEMPERATURE IN *PISUM SATIVUM L.*

Method

Seeds of four pea cultivars were soaked in incubators for 24 hours at 10 and at 30°C, in beakers full of distilled water. Respiration rate under nitrogen was measured in a Warburg apparatus as previously described, after gassing the reaction flasks for 10 minutes under nitrogen.

Results

Table 41 expresses the respiration rates under nitrogen as μl mg dry weight seed^{-1}hr^{-1} at 10 and at 30°C. The rate of increase over a 10 degree range is quoted as the Q_{10} (ie. square root of the 20 degree range measured).

Cultivar Meteor gave the lowest respiration rates under nitrogen at both 10 and at 30°C, but a higher Q_{10} over this range than the other species. Cultivars Gladstone, Onward and Pioneer increased their respiration rate under nitrogen in that order (at both 10 and 30°C) while their Q_{10} values decreased in the same order.

Table 42 gives a comparison of germination percentage (per cent germination when compared with control) after presoaking treatments of 24 hours at 10 and at 30°C with respiration rate

under nitrogen after presoaking for 24 hours at 10 and at 30°C.

Table 41.

Respiration of four pea cultivars under nitrogen after soaking seeds for 24 hours at 10 and at 30°C. (μl mg^{-1} dry weight seed $hour^{-1}$).

		Cultivar			
		Meteor	Onward	Pioneer	Gladstone
QCO_2^n $hr^{-1}mg^{-1}$					
	10°C	.0169	.0533	.0511	.0464
	30°C	.490	.621	.613	.564
Q_{10}		5.38	3.41	3.46	3.49

Where Q_{10} is given by

$$\left(\frac{QCO_2{}^n \ 30°C}{QCO_2{}^n \ 10°C} \right)^{10/\text{temperature}_1 - \text{temperature}_2}$$

For a 20° temperature range

$$\left(\frac{QCO_2{}^n \ 30°C}{QCO_2{}^n \ 10°C} \right)^{1/2}$$

or

$$\sqrt{\frac{QCO_2{}^n \ 30°C}{QCO_2{}^n \ 10°C}}$$

Table 42. Comparison of QCO_2^n at 10 and 30°C after 24 hours presoaking treatment of seed and germination after soaking seeds for 24 hours at 10 and at 30°C.

	Cultivar			
	Meteor	Onward	Pioneer	Gladstone
QCO_2^n $hr^{-1}mg^{-1}$ 10°C	.0169	.0533	.0511	.0464
germination % 10°C	88	22	0	58
$QCO_2^n hr^{-1}mg^{-1}$ 30°C	.490	.621	.613	.564
germination% 30°C	50	35	42	63

(all temperatures in the body of the table relate to the temperature of a 24 hour presoaking treatment). (Germination percentage is corrected with reference to the control treatments ie. no soaking).

III. CHANGES IN THE PROPERTIES OF THE ENZYME INVERTASE WITH TEMPERATURE OF SOAKING SEEDS OF PISUM SATIVUM L.

Invertase is known to increase its activity, or arise as a result of de novo synthesis during the germination of seeds (Eldon and Mayer, 1974); there are many reports relating to the role of invertase in the metabolism of carbohydrates in plant tissue (Gardner and Peel, 1971; Hatch and Glasziou, 1963; Hawker and Hatch, 1965; Jaiswal and Verma, 1971; Lyne and apRees, 1971; McLachlan, Datko, Rollit and Stokes, 1970; Ricardo and apRees, 1970; Seitz and Land, 1968) and Hawker (1971) demonstrated that invertase was the only enzyme catalysing sucrose breakdown.

During germination starch reserves are converted by amylase enzymes into soluble sugars (eg maltose.) prior to translocation to the meristematic regions (for model of starch brakdown seeDunn, 1974). The sugars then rapidly decline as germination proceeds (eg. Mayer and Poljakoff-Mayber, 1967) and also during soaking treatments (Chapter on sugar metabolism in Section A in current thesis).

A study of invertase activity will therefore, not only mirror sucrose changes, but will also clarify the enzymatic nature of sucrose breakdown in seeds during soaking treatments. This section

examines changes in the activity of this enzyme as a key to internal changes in the seed in relation to temperature and duration of presoaking treatment.

Method

Ten g of seed of each of four pea cultivars were soaked in distilled water (using 100 ml beakers in controlled temperature incubators in darkness) for 0, 12, 24 and 48 hours at 0, 10, 20 and 30°C in all combinations. They were then extracted in chilled Na_2HPO_4-citric acid buffer (pH 8.0) and passed through muslin before centrifugation for 30 minutes at 7, 000 rpm. The samples were then mixed with a known amount of sucrose solution and agitated in a warm water bath at 30°C. Samples were taken at two hourly intervals and estimated against standard thiosulphate (appendix).

To determine the optimum pH for pea seed invertase activity, a range of buffers from pH 4.6 to pH 8.6 was used to estimate specific activity of invertase in pea cultivar Meteor, after soaking seed for 12 hours at 20°C (Table 43 and Figure 29). The highest activities were recorded at pH 8.0 and all further invertase determinations were carried out using a buffer of pH 8.0

Results

Specific activities of invertase (I.U. mg protein^{-1} x 10^{-5}) are given in Table 44, and activities per g dry weight seed (I.U. g dry weight seed^{-1}) are given in Table 45. The effect of temperature on invertase was small and probably not significant, in all except the longest soaking treatments (48 hours). In this treatment both specific activities and activity per g seed rose rapidly with a rise in temperature. This rise was more marked in cultivars Pioneer and Onward (1.1 and 1.4 I.U. g dry weight seed^{-1} respectively) compared with cultivars Meteor and Gladstone (.84 and .26 I.U. g dry weight seed^{-1}).

Figures 30 to 33 show specific activities for invertase and Figures 34 to 37 show activity per g seed in graph form.

Similarly the duration of soaking treatment showed little effect on invertase activity after 12 or 24 hours of soaking, but after 48 hours of soaking invertase activity rose in pea seeds with temperature.

Table 43.

Specific activities for Meteor pea seed invertase after soaking seeds for 12 hours at 25°C using buffers of varying pH for the extraction.

	pH of buffer					
	4.6	5.6	6.6	7.6	8.0	8.6
Specific activity of invertase (I.U.mg protein^{-1})	0	.00011	.00010	.00053	.013	.0049

Figure 29. The relationship between buffer pH and invertase specific activity in Meteor pea after soaking seed for 12 hours at 25°C.

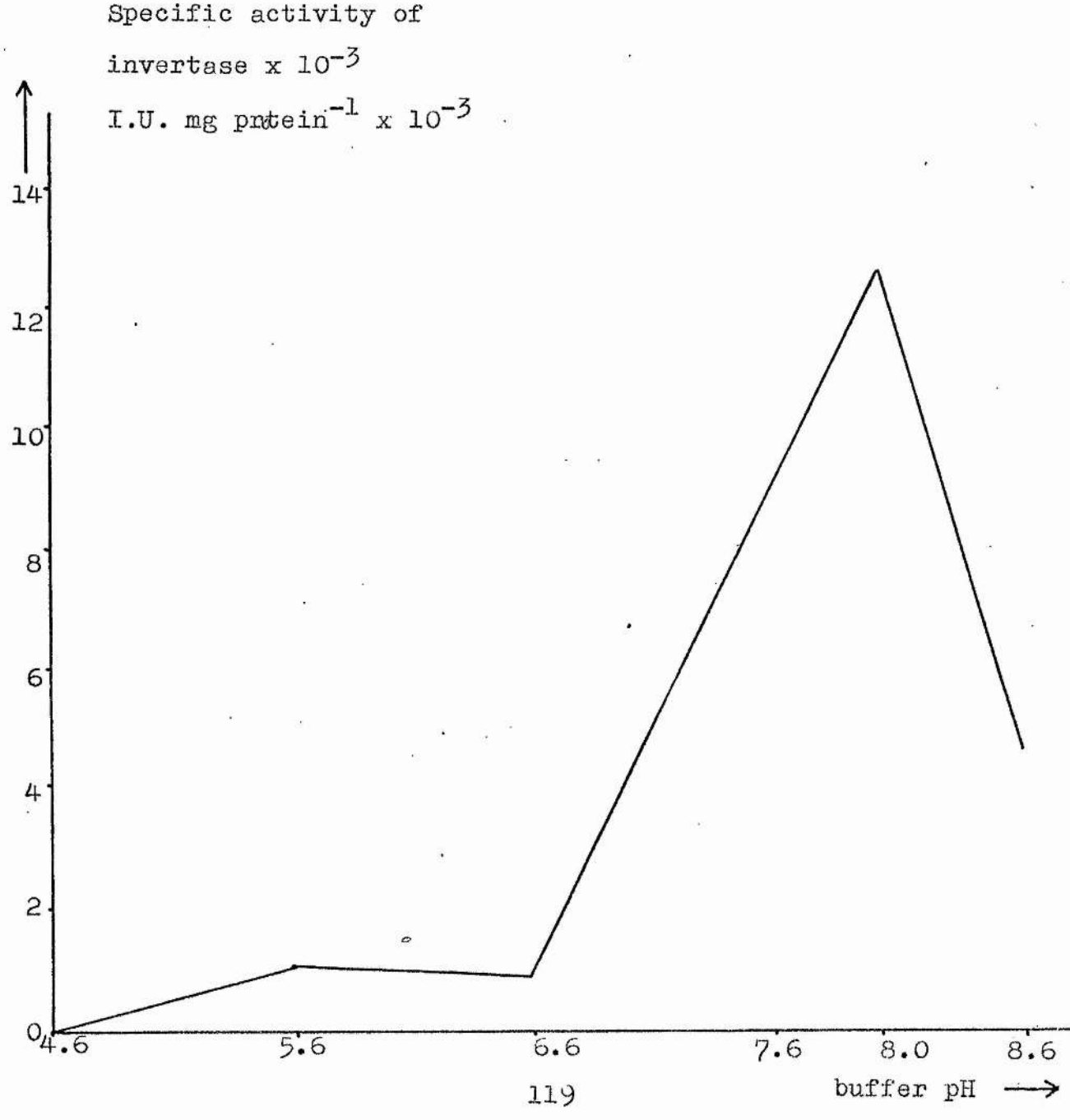


Table 44.

Invertase specific activity (I.U. mg protein^{-1} x 10^{-5}) of pea seed extracts after soaking seeds from 0 to 48 hours at 0 to 30°C.

		Temperature of soaking			
Cultivar	Hours soak	0	10	20	30°C
Meteor	none	1.60			
	12	5.68	4.38	5.19	3.40
	24	2.94	2.39	1.60	2.41
	48	3.12	5.17	21.0	31.3
Onward	none	.410			
	12	4.83	5.47	7.71	3.40
	24	6.27	2.83	1.53	1.80
	48	5.45	7.14	51.1	45.8
Pioneer	none	1.10			
	12	1.42	3.20	7.68	23.4
	24	5.63	3.63	4.01	.900
	48	1.68	6.67	48.4	45.0
Gladstone	none	.240			
	12	8.40	3.80	4.90	17.6
	24	5.29	5.35	7.53	.833
	48	5.65	6.50	94.9	19.1

Table 45.

Invertase I.U. g dry weight seed^{-1} of pea seed extracts after soaking treatments from 0 to 48 hours at 0 to 30°C.

		Temperature of soaking			
Cultivar	Hours soak	0	10	20	30°C
Meteor	none	.0300			
	12	.0286	.0473	.0433	.0729
	24	.0276	.0356	.0340	.0562
	48	.0530	.0910	.840	.840
Onward	none	.0160			
	12	.0365	.0710	.106	.0867
	24	.0690	.0374	.0551	.0483
	48	.108	.140	1.42	1.42
Pioneer	none	,0330			
	12	.00985	.0197	.0630	.169
	24	.0788	.0493	.0324	.0374
	48	.0240	.116	1.20	1.11
Gladstone	none	.00790			
	12	.0532	.0236	.0510	.171
	24	.0709	.0749	.0543	.0315
	48	.0990	.134	1.39	.256

Figure 30. Change in specific activity of invertase with temperature and duration of soaking treatment in seeds of pea cultivar Pioneer.

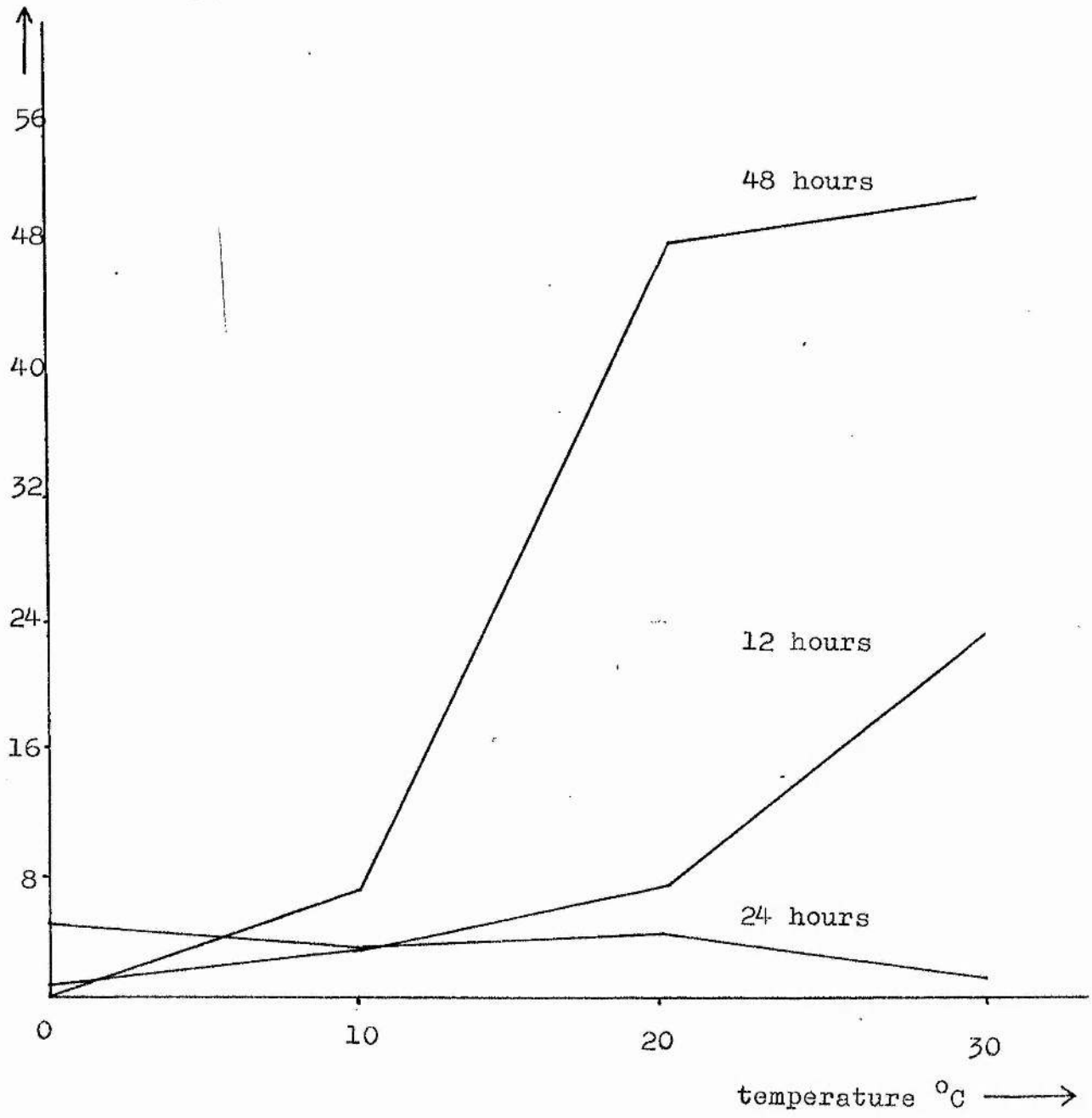


Figure 31. Change in specific activity of invertase with temperature and duration of soaking treatment in seeds of pea cultivar Meteor.

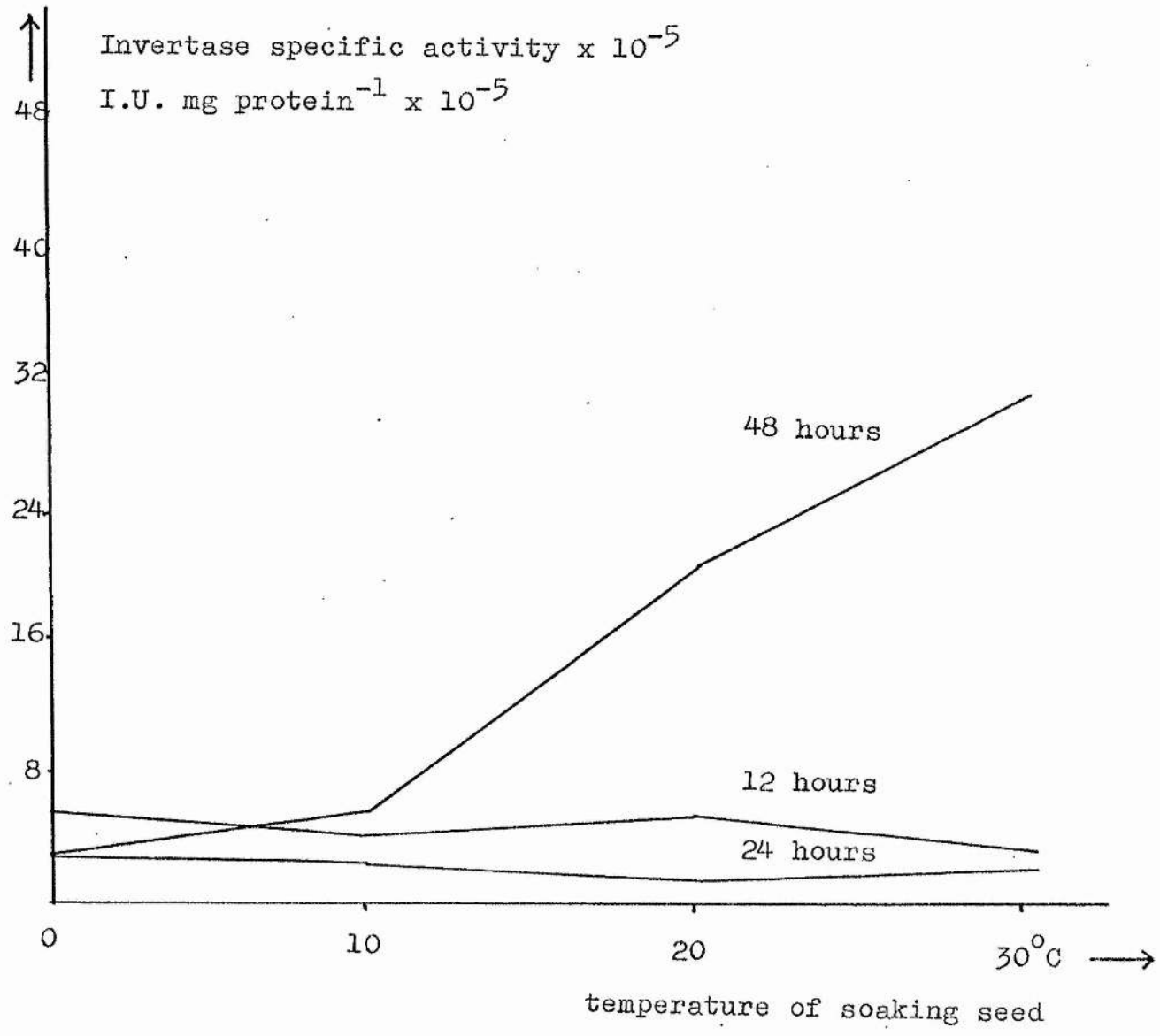


Figure 32. Change in specific activity of invertase with temperature and duration of soaking treatment in seeds of pea c ultivar Onward.

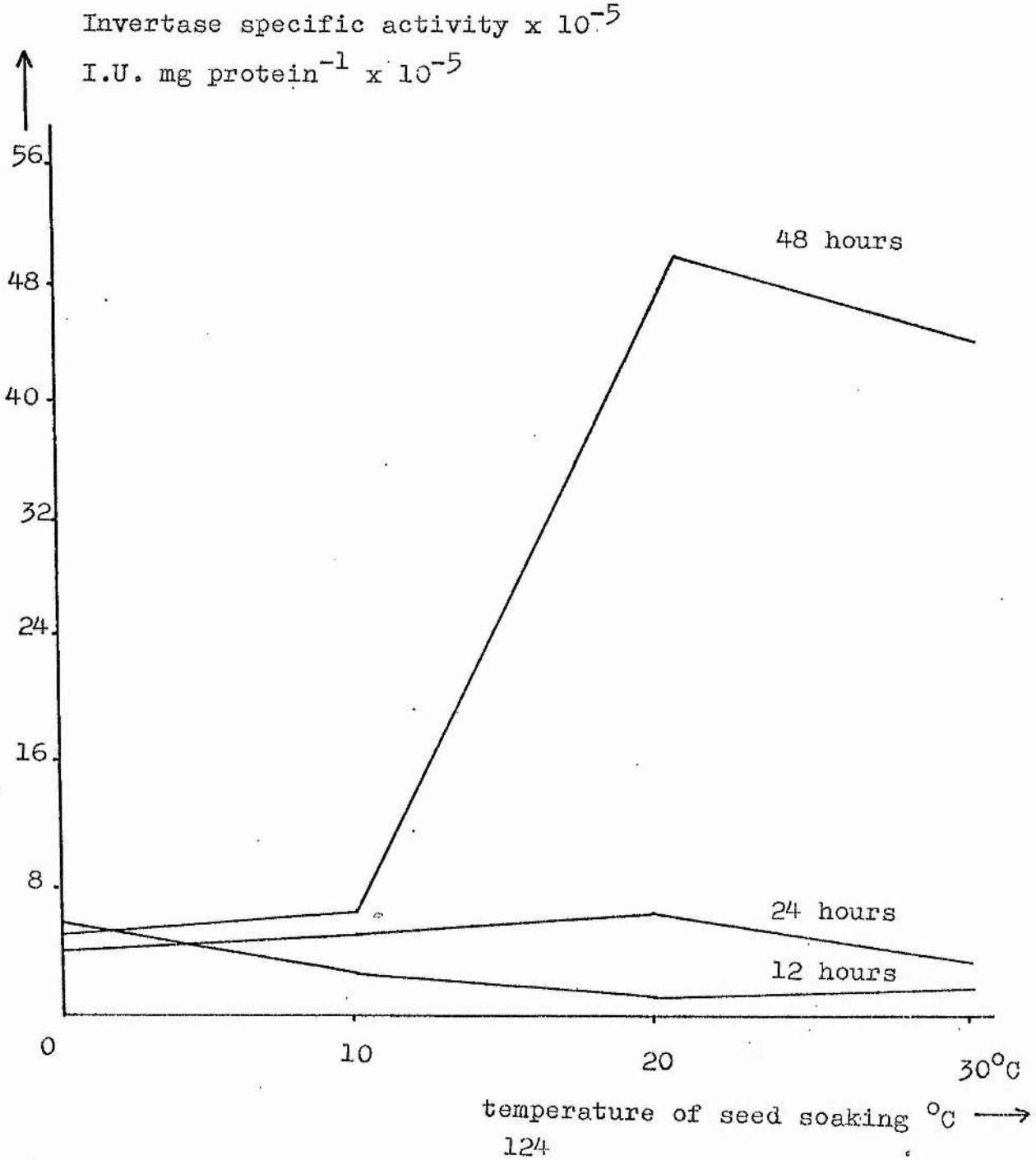


Figure 33. Change in specific activity of invertase with temperature and duration of soaking treatment in pea cultivar Gladstone.

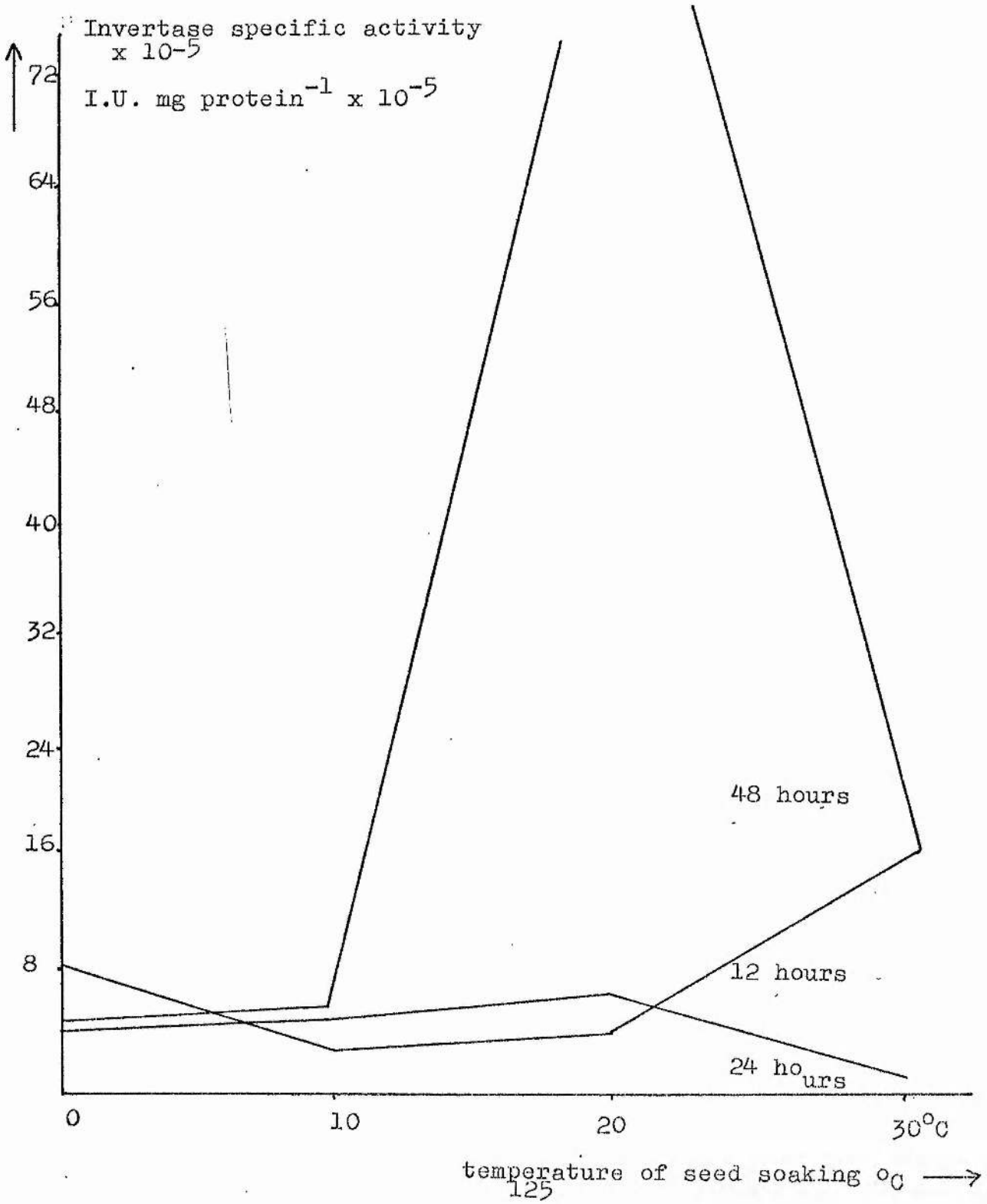


Figure 34. Change in invertase I.U. g dry weight seed^{-1} after soaking treatments from 0 to 48 hours at 0 to 30°C in Meteor pea.

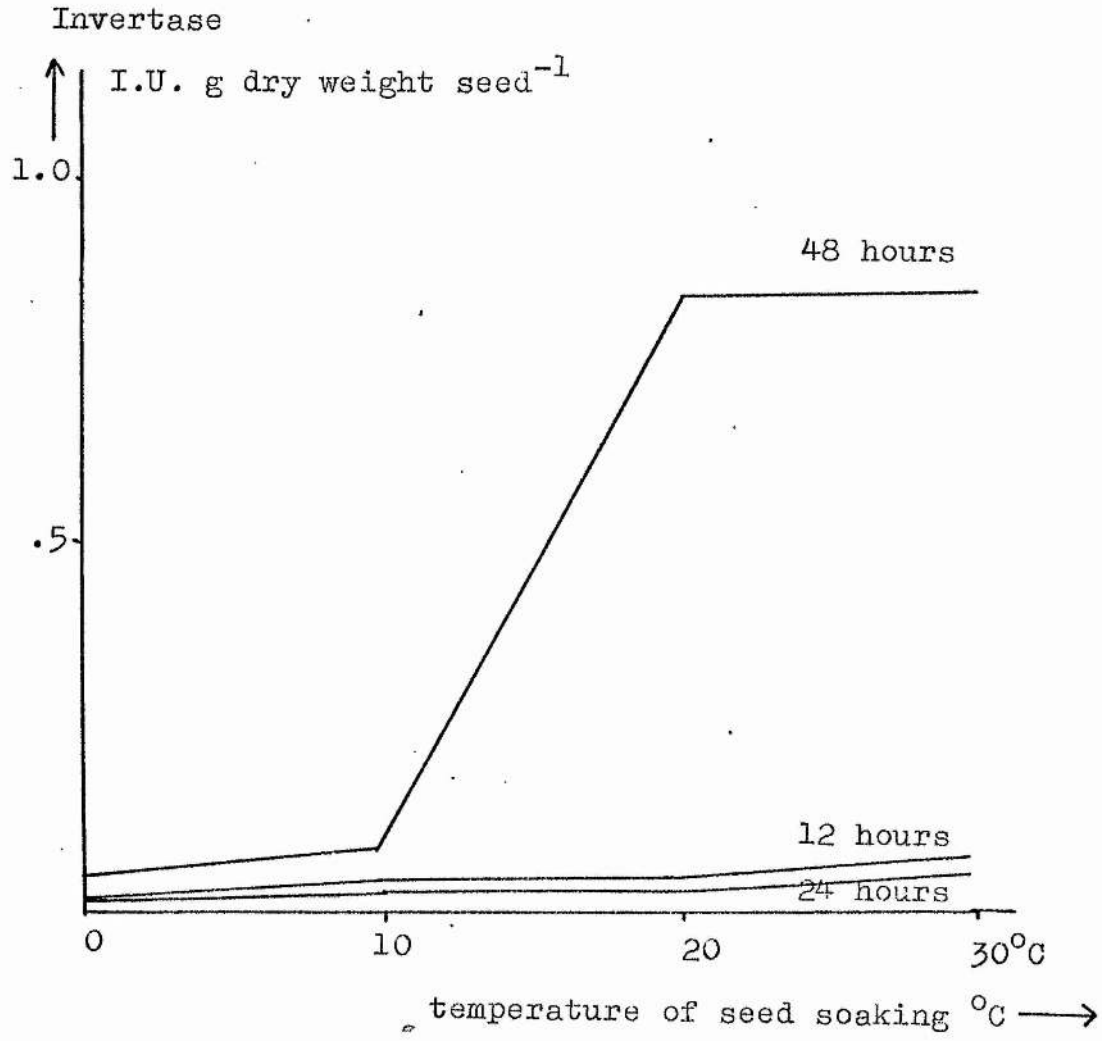


Figure 35. Change in invertase I.U. g dry weight seed^{-1} after soaking treatments from 0 to 48 hours at 0 to 30°C in Pioneer pea.

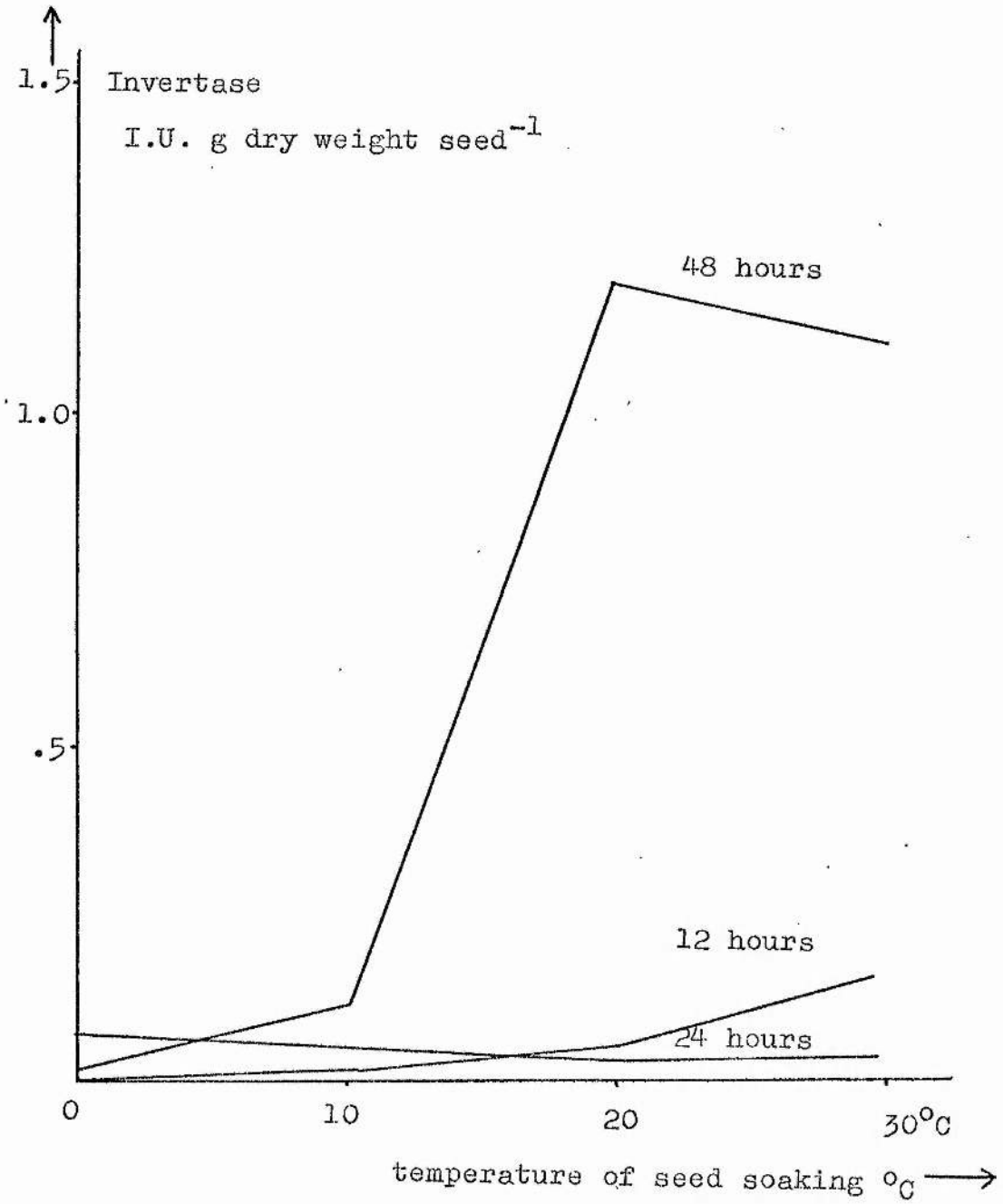


Figure 36. Change in invertase I.U. g dry weight seed^{-1} after soaking treatments from 0 to 48 hours at 0 to 30°C in Onward pea.

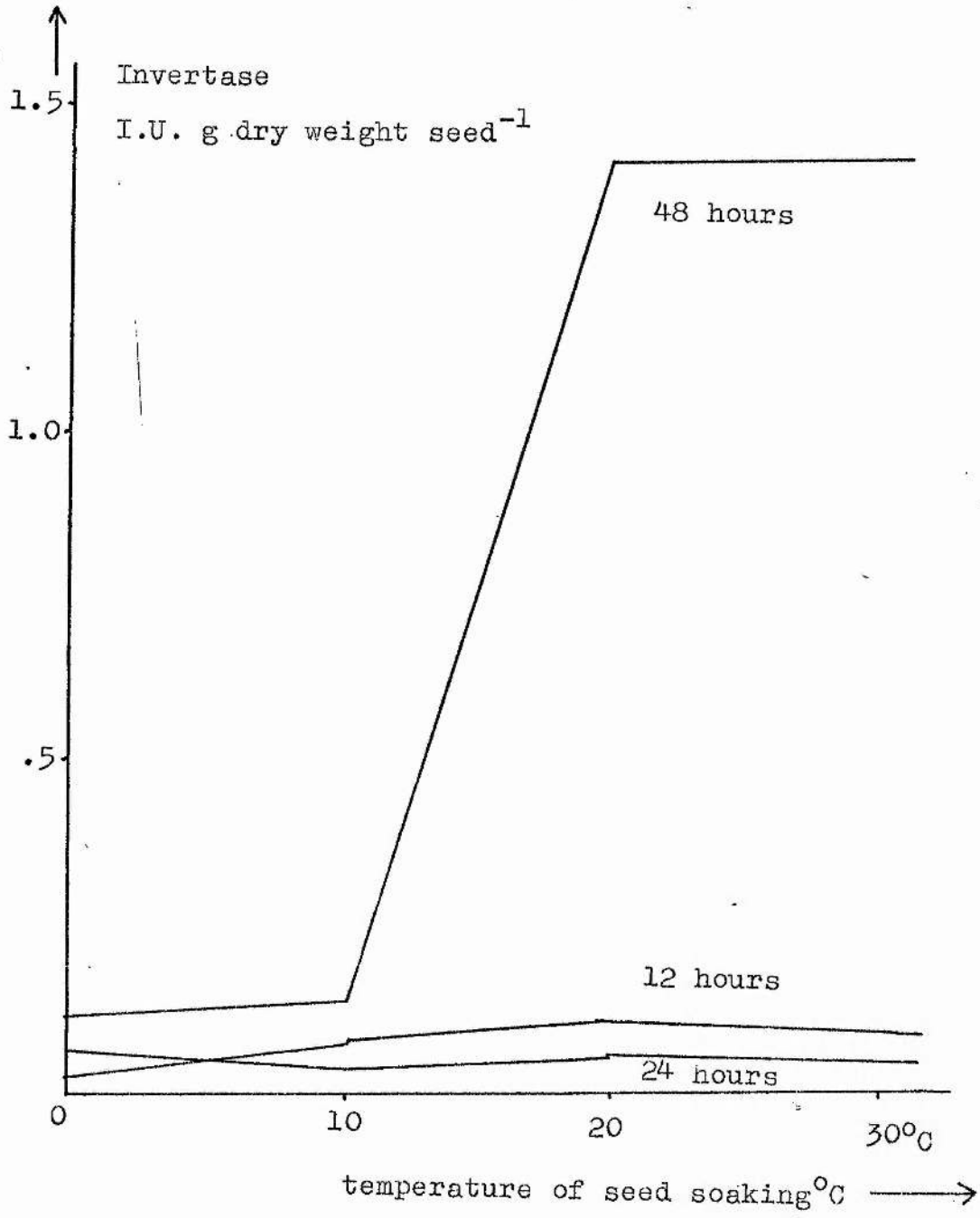
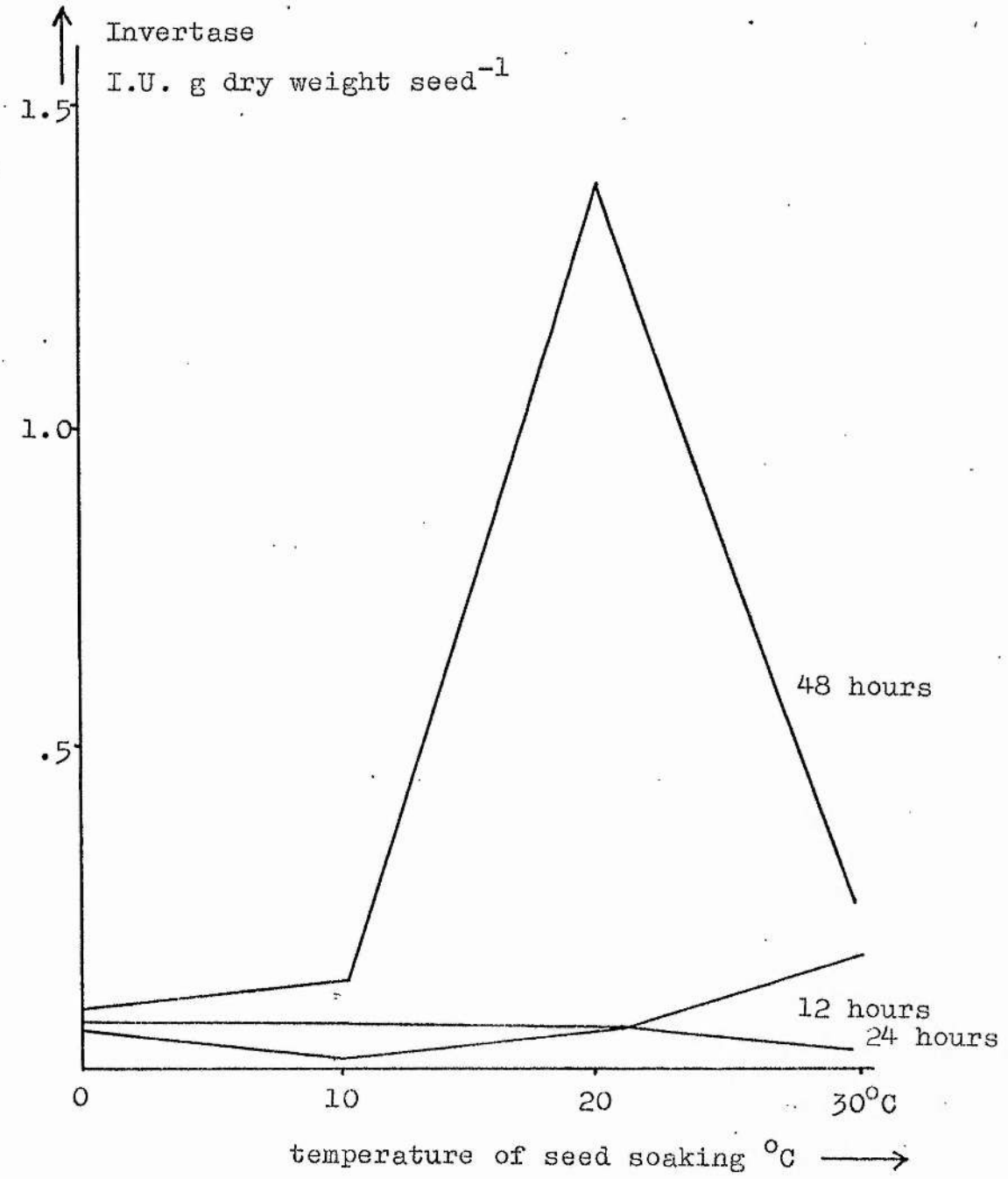


Figure 37. Change in invertase I.U. g dry weight seed^{-1} after soaking treatments from 0 to 48 hours at 0 to 30°C in Gladstone pea.



IV. CHANGES IN THE ACCUMULATION OF END PRODUCTS OF ANAEROBIOSIS WITH TEMPERATURE IN PISUM SATIVUM L.

The accumulation of alcohol and malate in pea seeds in relation to temperature of soaking.

The rate of a metabolic reaction will normally rise with temperature (Q_{10} effect) and therefore anaerobic respiration should rise with an increase in temperature if seeds are germinated under partial anoxia. Thus if the rate of glycolysis increases then the end products of respiration will also increase.

A study of the relative levels of these end- products and their accumulation in relation to temperature will show the extent of damage occurring within the seed. In this section four pea cultivars (of different soaking tolerance) are compared with reference to temperature effects on alcohol and malic acid accumulation.

Method

A known weight of seed of each of four pea cultivars (Meteor, Onward, Pioneer and Gladstone) were soaked underwater for 24 hours at 0, 6, 10, 20 and 30°C in incubators and assayed for alcohol and malic acid as previously described (see also appendix).

Results

The effect of temperature of soaking on alcohol and malic acid in pea seeds is expressed as μM g dry weight seed^{-1} in Table 46. Table 47 shows regression anlyses for ethanol and Table 48 for malic acid against temperature of soaking in the four pea cultivars.

All pea cultivars fell marginally short of significance for ethanol against temperature of soaking treatment, except Gladstone ($p=.01$), however, the data suggest that the largest rise is from 20 to 30°C and therefore the relationship is not necessarily linear. For malic acid only Pioneer gave a significant linear regression ($p=.025$) with temperature of soaking treatment; the regression was negatively correlated.

Figures 38 and 39 show the effect of temperature on alcohol and malic acid respectively with 95% confidence intervals marked at 30°C (at lower temperatures there was no significant difference). Gladstone and Meteor yielded higher malic acid content than Pioneer and Onward, while Pioneer gave the highest alcohol content at 30°C although the differences at other temperatures were not significant.

Table 49 gives a summary of alcohol and malic acid content of seeds at 30°C in relation to germination percentage at 30°C.

Table 46. The effect of temperature of a 24 hour soaking treatment on ethanol and malic acid levels in the seeds of four pea cultivars (μM g dry weight seed^{-1}).

Cultivar		Temperature of soaking				
		0	6	10	20	30°C
Meteor	alcohol	54.630	61.686	69.936	71.525	159.308
	malate	2.690	3.138	3.425	4.403	2.911
Onward	alcohol	68.029	74.603	79.057	89.355	185.470
	malate	1.562	.873	.469	1.092	1.094
Pioneer	alcohol	89.942	100.900	109.070	118.153	329.672
	malate	3.542	3.537	3.660	2.478	1.180
Gladstone	alcohol	63.380	84.198	97.290	107.787	137.393
	malate	3.906	2.682	1.885	1.749	2.961

Each value in the body of the table is the mean of three replicate samples.

Table 47. Regression analyses for ethanol (μM g dry weight seed^{-1}) against temperature (0, 6, 10, 20 and 30°C) of 24 hour soaking treatment in the seeds of four pea cultivars.

Source of variation		Sum of squares	Degrees freedom	Mean square	F
Meteor	Regression	5570.092	1	5570.092	9.215
	Residual	1813.453	3	604.684	
	Total	7383.543	4		N.S.
Onward	Regression	7335.103	1	7335.103	10.063
	Residual	2186.665	3	728.888	
	Total	9521.768	4		N.S.
Pioneer	Regression	29461.410	1	29461.410	7.668
	Residual	11526.859	3	3842.286	N.S.
	Total	40988.268	4		
Gladstone	Regression	2932.594	1	2932.594	84.154
	Residual	104.544	3	34.848	
	Total	3037.137	4		p=.01

Table 48. Regression anlyses for malic acid (μM g dry weight seed^{-1}) against temperature (0, 6, 10, 20 and 30°C) of 24 hour soaking treatment in the seeds of four pea cultivars.

Source of variation		Sum of squares	Degrees freedom	Mean square	F
Meteor	Regression	.1849	1	.1849	.3521
	Residual	1.5756	3	.5232	
	Total	1.7606	4		N.S.
Onward	Regression	.004720	1	.004720	.01838
	Residual	.7706	3	.2569	
	Total	.7753	4		N.S.
Pioneer	Regression	3.9542	1	3.9542	20.608
	Residual	.5756	3	.1919	
	Total	4.5299	4		p=.025
Gladstone	Regression	.4125	1	.4125	.4564
	Residual	2.6589	3	.8863	
	Total	3.0714	4		N.S.

Figure 38. The effect of temperature of a 24 hour soaking treatment on alcohol content of seeds of four pea cultivars with 95% confidence intervals marked for 30°C.

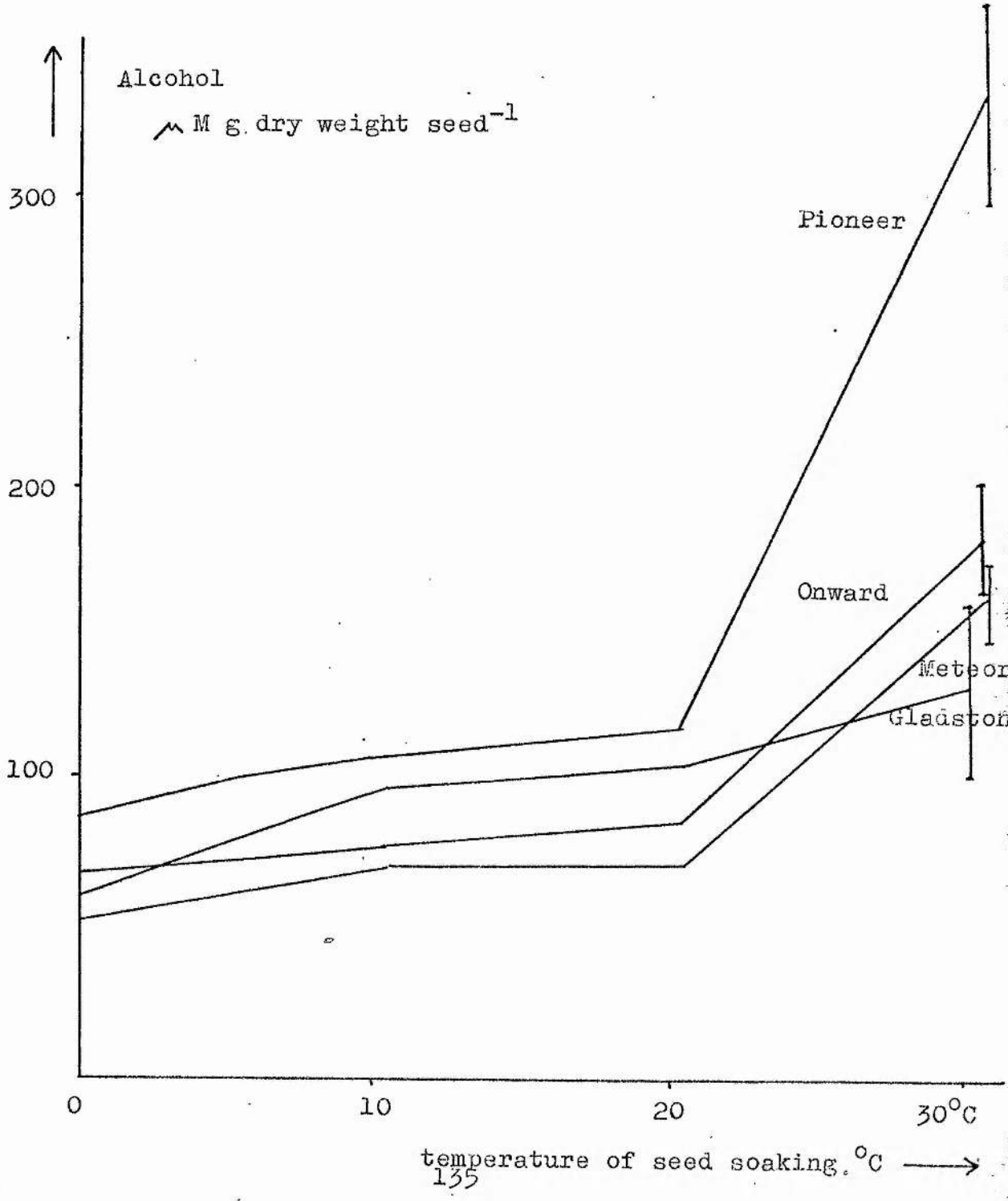


Figure 39. The effect of temperature of a 24 hour soaking treatment on malic acid content of seeds of four pea cultivars with 95% confidence intervals marked for 30°C.

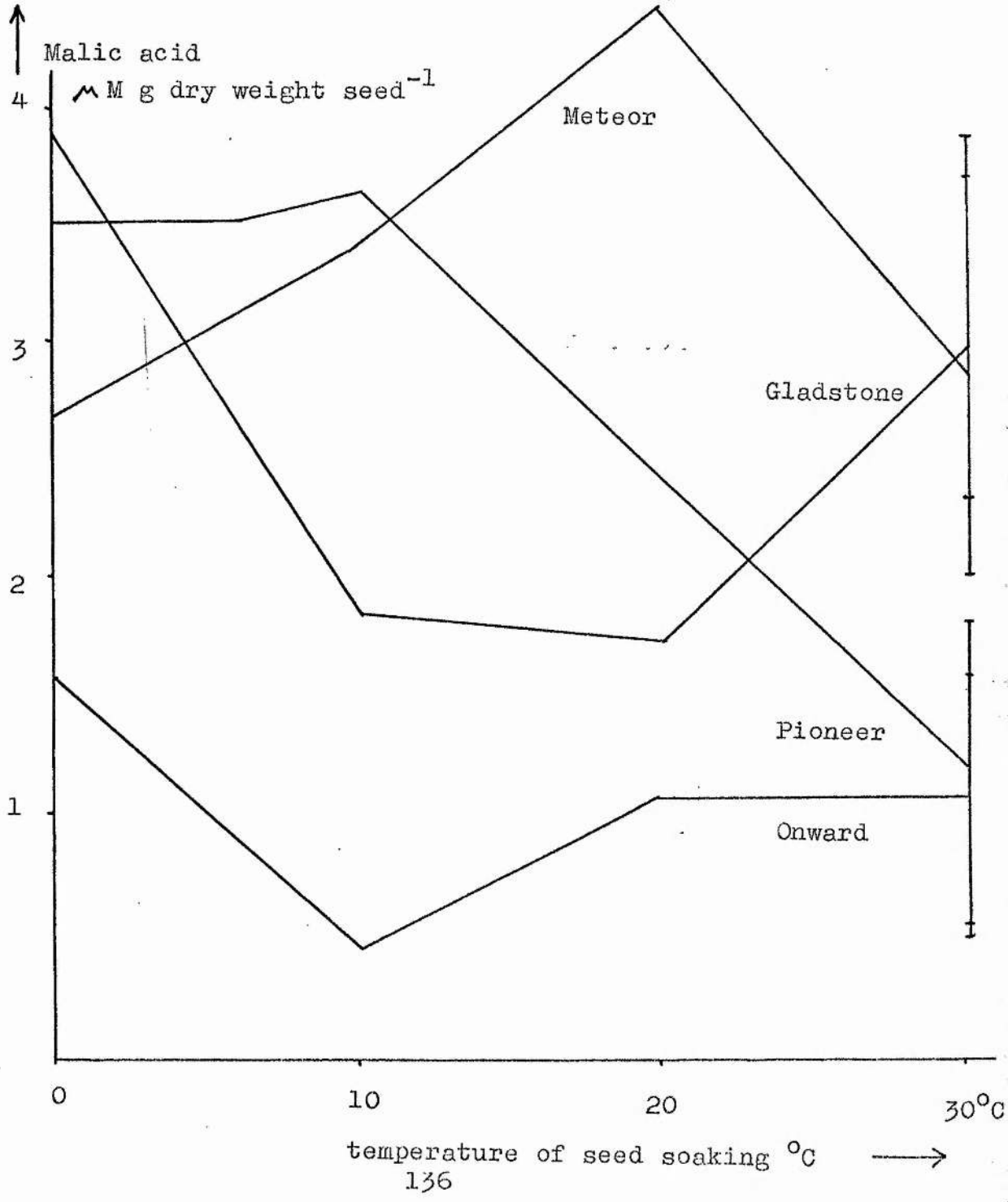


Table 49. Comparison of alcohol and malic acid accumulation (μM g dry weight seed^{-1}) at 30°C with the fall in germination observed when seeds of four pea cultivars were soaked for 24 hours at 30°C.

Cultivar	% germination after 24 hrs 30°C soak compared with control.	30°C alcohol accumulation (μM g dry weight seed^{-1}).	30°C malate accumulation (μM g dry weight seed^{-1}).
Meteor	50%	159.3	2.9
Gladstone	63%	137.4	3.0
Onward	35%	185.5	1.1
Pioneer	42%	329.7	1.2

C. DISCUSSION

I. Introduction

A striking range of tolerance to injury was observed when seeds were germinated underwater. Some species (rice, radish and carrot) germinated equally well under water as in controls; others (lettuce) germinated slighlty less well underwater; while some species completely failed to germinate under water (maize, broad bean and four pea cultivars)(Table 1).

The nature of this difference, whether resulting from the physical environment of the seed or its internal metabolism, has not previously been investigated in seeds, although damage due to waterlogging has frequently been observed in the poor field emergence of a number of crops.

Previous workers on flooding tolerance have studied the grown plant (eg. Crawford and co-workers) where the presence of an aerial part of the plant may complicate any changes which occur. For example, Armstrong (1964) demonstrated the outward diffusion of oxygen from roots otherwise under partial anoxia. Similarly the tolerance of flooding in rice has been largely attributed to the ability to diffuse oxygen from the roots to the shoots and into an anaerobic surrounding (van Raalte, 1940). However,

the seed represents a simplification of this process and controlled experiments on its metabolism are easily performed using controlled temperature incubators in darkness.

Soaking injury under field conditions

This damage is often observed as secondary infection which is usually associated with waterlogging, but this is probably symptomatic of internal damage to the seed, which results in a lowered resistance to infection. Evidence for this may be taken from the poor correlation between laboratory germination tests and field emergence of seedlings (Perry, 1970) and thus some factor other than viability must be important in pre emergence mortality. Support for this argument is given by Heydecker (1968) who proposed that the sugar content of seeds is important as a determinant of microbial colonization and that the possible leakage of sugars from the seed (Mathews and Garver, 1971) may actively encourage pathogen attack (Hayman, 1969).

Indeed a significant correlation for the electrical conductivity of steep water and field germination has been shown in castor beans (Thomas, 1960) and in peas (Mathews and Whitbread, 1968). Several workers have also found a correlation between seed exudation and infection by fungal pathogens

(Barton, 1957; Thomas, 1960; Flentje and Saksena, 1964).

However, although the ultimate cause of seedling death, under wet conditions in the field, may be due to secondary infection, the primary cause will be resultant from the physical and metabolic effects of submergence, which in turn lead to exudation from the seed as observed by other workers.

II. The nature of soaking injury

This thesis, therefore seeks to determine the nature of primary damage to the seed as a result of the physical and metabolic effects of submergence. A range of crop species has been chosen for this because seed was easily obtainable in uniform batches and also because field damage has frequently been reported in crops, and therefore this study has potential economic implications to agriculture.

The importance of oxygen

The lack of oxygen in the soakwater may account to some extent for the different behaviour between species, because many of the smaller seeded species showed greater tolerance to soaking in these experiments (Table 1), possibly because of a lower total oxygen requirement (which could be satisfied by the small amount of oxygen dissolved in the

soakwater). However, if the difference is a reflection of seed size alone then seeds of a similar size should show similar tolerance. This was not found to be the case when seeds of different cultivars of Pisum sativum L. were examined. A distinct difference in soaking tolerance was demonstrated (Table 2). This precludes the possibility that seed size alone controls soaking tolerance.

A second possible explanation is that tolerant seeds are able to germinate in the total absence of oxygen. However, several workers have shown that germination is not possible in the absence of oxygen (eg. James and Hora, 1940), but for some time it was thought that rice was an exception to this rule, but more recently this has been disproven. Kordan (1972) found that exposure of seedlings to low oxygen concentrations resulted in suspension of normal growth.

The effect of complete anoxia was again examined in these experiments on the range of species under study. When seeds were placed in anaerobe jars no germination was recorded in any species, which confirms a minimal oxygen requirement even in the most soaking tolerant species.

A further possibility is that of anaerobic induced dormancy as a result of the soaking treatments. To ascertain whether the species which

failed to germinate underwater, retained their germination capacity, an experiment was conducted on germination after a presowing soaking treatment of varying severity (from 0 to 96 hours seed soaking)(Table 2). Those species which germinated well underwater were not affected significantly by the presoaking treatments, but those species which were unable to germinate under water were markedly affected by the duration of presoaking. The intolerant species all gave a significant negative correlation for germination against hours of presoaking ($p=.05$ to $p=.01$) (Table 2), while the tolerant species did not give a significant correlation. This confirms that the ability to germinate underwater is not just a question of anaerobic induced dormancy.

Change in morphology of seedlings after soaking seeds

An indirect result of soaking injury in seeds might be a change in the morphology of the seedlings, such as height, but no correlation of height and duration of presoaking was shown in these experiments, however, there were relatively few degrees of freedom (2 to 3)(Table 5).

There is evidence in the literature for deleterious effects in grown plants raised from seed which has been soaked underwater. Kidd and

West (1919) showed that soaking seeds could encourage more rapid germination but that the yield was lower from such plants. Berrie (1960) found that on germination abnormal pea plants were common after soaking seeds prior to germination. Several alternative theories were proposed to explain this -

a. change in the normal metabolism
b. an essential metabolite is leached out
c. an adverse material is accumulated in the seed.

The changes observed were a supression of the terminal bud, development of basal auxillary buds and the leaf shape was affected. Treated plants were usually slightly chlorotic. These changes were probably resultant from damage to the meristem tissues due to anaerobic conditions. Similarly, Polya (1961) observed abnormalities in Populus alba seedlings after soaking seeds. The abnormalities observed included less firm rooting, imperfect geotropism and inverse germination.

III. METABOLIC REACTIONS TO ANOXIA

Changes in respiration rate

It is likely that any metabolic change on soaking will be reflected in the respiration rate of seeds. During imbibition respiration rates rise in seeds and in the early hours of germination respiration rates are related to the water content of seeds (Opik and Simon, 1963). At the same time seeds of high vigour have higher rates of oxygen uptake, than seeds of low vigour which is used as a basis of a respiratory test for seed vigour in peas (Woodstock, 1965). Under anoxia anaerobic respiration is likely to increase as a result of the need to satisfy the energy requirement of the seed (discussed later).

The effect of soaking seeds on their gaseous exchange was studied using standard manometric techniques, in some detail in rice (highly tolerant of soaking injury), in Meteor pea (of intermediate soaking tolerance) and in Pioneer pea of poor soaking tolerance). The highest metabolic rates were observed in the species least tolerant of soaking injury. The respiratory quotient was highest in rice followed by Pioneer and Meteor pea ($p=.001$, Table 10), but a significant increase in RQ was observed in all three species on soaking

compared with controls (Table 11). This indicates a relative increase of carbon dioxide evolution to oxygen uptake ie. due to lack of available oxygen, uptake will fall but carbon dioxide continues to be evolved resulting in the observed change in RQ. Indeed, in the two pea cultivars there was a significant increase in carbon dioxide evolution on soaking ($p=.02$ to $p=.001$)(Table 11), while there was no significant change in rice.

Fluctuation in carbon dioxide evolution rates are common in the normal germination of seeds (Spragg and Yemm, 1959) and it is therefore not necessarily a measure of glycolysis, since fluctuations in RQ may suggest the oxidation of highly reduced metabolites eg. ethanol oxidation which may take place on the change from an anaerobic to an aerobic environment (Peterson and Cossins, 1966; Cossins and Turner, 1963). This change from anaerobiosis to aerobiosis occurs naturally as the seed coat is ruptured by the emerging radicle (eg. Cossins and Turner, 1963). Similarly this change may also occur to some extent underwater, when the seedcoat is ruptured there may be greater access of oxygen from the soakwater if it has not already been depleted.

However, injurious treatments to seeds resulting in differences to vigour are usually

reflected by differences in respiration rates of seeds during the first few hours of imbibition eg. sorghum, radish and wheat (Woodstock and Justice, 1967) and in lima beans (Woodstock and Pollock, 1965).

Respiration under nitrogen was highest in Pioneer pea, followed by Meteor pea and rice (Table 10)($p=.001$) ie. the lowest values were recorded in the species best able to germinate underwater. The nature of the change of respiration under nitrogen differed between species. In Pioneer pea there was a significant negative correlation ($p=.01$, Figure 4) for respiration under nitrogen with duration of soaking treatment, which suggests severe substrate inhibition because of the high overall anaerobic respiration rate (Table 10). In Meteor pea a significant positive correlation was given ($p=.01$, Figure 3) ie. respiration continues rapidly but at a slightly lower overall rate than in cv. Pioneer and can thus satisfy the energy requirement of the seed prior to seizure of the reaction due to substrate inhibition. In rice there was no significant correlation of respiration under nitrogen with duration of soaking treatment, which suggests that anaerobic metabolism is unchanged as a result of soaking in this species.

It therefore is likely that the absolute rate

of respiration under nitrogen is correlated with the survival ability of seeds underwater. To this end respiration and respiration under nitrogen were measured in several other species.

Table 14 demonstrates the relationship between respiration under nitrogen and species germination ability under water. This suggests that germination classes of seeds under anoxia mirror their metabolic classes as measured by respiration under nitrogen. To establish this relationship a regression analysis for the absolute rate of respiration under nitrogen (after a 24 hour presoaking treatment) against the fall in germination after a 72 hour presoaking treatment of the seeds of each species was carried out (this soaking treatment was chosen so as to give good differentiation between species) (Table 50 and Figure 40).

This analysis establishes a significant negative correlation ($p=.01$) for QCO_2^n against germination after soaking seeds. There appear to be two classes of interaction between QCO_2^n and germination. Where species with a ratio of germination after a 72 hour presoak to control germination exceed unity (ie. a 72 hour soaking treatment does not have a deleterious effect on germination) form a separate class from the intolerant group (Figure 40).

Table 50. The relationship between the decrease in germination after a 72 hour presoaking treatment (ratio of number germinated after a 72 hour presoaking treatment to the number germinated after no presoaking treatment) and their respiration rate under nitrogen (μ l mg dry weight^{-1} hr^{-1}) in the seeds of some crop species.

Species or cultivar	Ratio number germinated after 72 hr presoak: no presoak	Respiration under nitrogen (μ l mg dry weight^{-1} hr^{-1})
Pioneer pea	0	.569
Meteor pea	0	.445
broad bean	.5556	.269
maize	.6667	.240
lettuce	1.0147	.199
rice	1.7500	.103
radish	1.0625	.0616
carrot	1.3077	.0263

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	.2013	1	.2013	25.302
Residual	.04773	6	.007955	
Total	.2490	7		p=.01

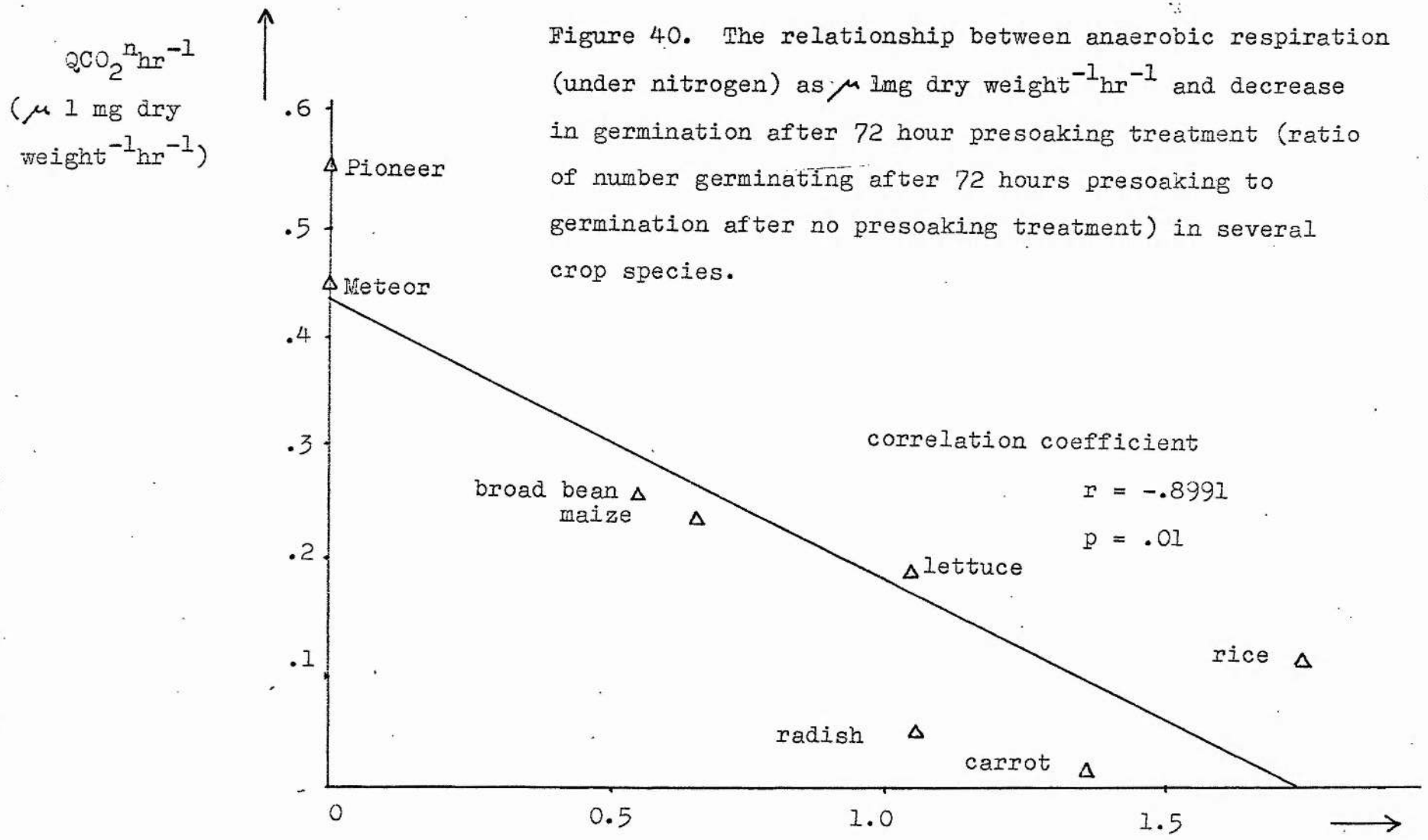


Figure 40. The relationship between anaerobic respiration (under nitrogen) as μl mg dry weight^{-1}hr^{-1} and decrease in germination after 72 hour presoaking treatment (ratio of number germinating after 72 hours presoaking to germination after no presoaking treatment) in several crop species.

The adoption of anaerobic pathways under anoxia has been reported many times in the literature, however anaerobic respiration is far less efficient than oxidative phosphorylation. Considering glucose as the source of energy, we find that the common pathway (discussed later) two molecules of ATP are generated for each molecule of glucose consumed. If metabolism is then aerobic NADH will then be oxidised (three molecules of ATP times two) and finally decarboxylation via the citric acid cycle will yield more ATP (two molecules times fifteen). In summation therefore, for each molecule of glucose consumed thirty eight molecules of ATP will be generated in aerobic metabolism while only two molecules of ATP are generated in anaerobic metabolism viz. aerobic respiration produces nineteen times as much metabolic energy. It is notable, probably for this reason, that very few, if any, organisms are able to maintain a solely anaerobic process for any length of time.

Tables 51 and 52 summarise some of the important observations of anaerobiosis and their usual duration. From these tables it can be seen that organisms either undergo short term anaerobic processes accumulating non toxic end products or they have an efficient disposal system such as

excretion (in animals) or translocation to aerobic surroundings (eg. the aerial part of the plant where the roots are flooded) or transport from partially anaerobic to aerobic parts of the organism (eg. where tissue is very compact).

Thus it is highly probable that seeds sown under flooded conditions will be unable to survive anaerobiosis for more than a few hours or days, because excretion (other than exudation from the seed) or transport of toxic materials to aerial portions of the plant is not possible.

Differences in tolerance of seeds are likely to result from differences in anaerobic respiration rate and hence accumulation of toxic products or in the differential accumulation of non toxic products. At the same time a high QCO_2^n will impart a higher metabolic rate which will be reflected in a change in the storage products of the seed.

Table 51. The duration and tolerance of anerobiosis in several organisms where anaerobic respiration is of frequent occurrence.

Organism	Main end product (toxic)	Disposal (damage to organism)	Duration anaerobiosis	Reference
ANIMAL				
Diving seal	lactate (no)	remetabolism (no)	minutes	Bryant,1971
Human sprinter	lactate (no)	remetabolism (no)	minutes	Bryant, 1971
Cockroach	lactate (no)	remetabolism (no)	5-6hours	James,1971
Ascaris nematode	succinic acid(no)	excretion (no)	5-6 days	James,1971
African Trypanosomes	lactate + glycerol (no)	excretion (no)	always	vonBrand,1966
Diapausing silkworm eggs	glycerol (no)	remetabolism (no)	days/weeks	Gilmour,1965
Moliniformis	alcohol (yes)	excreted (no)	always	James,1971

Table 52. The duration and tolerance of anaerobiosis in several plant species where anaerobic respiration is of frequent occurrence.

Organism	Main end product (toxic)	Disposal (damage to organism)	Duration anaerobiosis	Reference
PLANT				
Potatoes	lactate (no)	remetabolism (no)	days/weeks	James, 1953
Alnus	glycerol (no)	remetabolism (no)	days/weeks	Crawford, 1972
Yeasts	alcohol (yes)	diffusion into growth medium (eventually ceases growth)	days/weeks	Pasteur, 1880
Iris	shikkimate (no)	transport in rising sap? (no)	days/weeks	Tyler and Crawford, 1970
Betula	malate (no)	in rising sap? (no)	days/weeks	Crawford, 1972

Changes in storage materials on soaking seeds

On germination storage materials are metabolised partly to provide energy during their breakdown, and partly to provide building blocks from which macromolecules may be synthesised for growth of the seedling. The level of destructive breakdown of reserve materials will therefore depend upon the efficiency of energy use and the rate of growth. Because anaerobic respiration results in the loss of more free energy and avoids the enrgy rich oxidative phosphorylation steps of the Krebs cycle, germination of seeds underwater is likely to result in greater destruction of reserves for energy consumption than in normal germination.

It should be emphasised that starch is the main storage material in a number of seeds but this is normally converted (usually by the amylase enzymes eg. in peas, Juliano and Varner, 1969; in rice, Murata etal., 1968) to mobile compounds (sugars) to facilitate translocation to the meristematic regions. The major soluble carbohydrate is sucrose in rice (Murata etal., 1968), in lettuce (Mayer and Poljakoff-Mayber, 1967) as well as in many other seeds and this declines during the first few days of germination

as it is transferred to the growing point of the seedling. Also large amounts of glucose (which was formed directly from starch by α amylase) and fructose have been reported in a number of species eg. in rice (Murata, etal., 1968) and in Phaseolus (Morohashi and Shimokoriyama, 1972).

In order to monitor changes in storage materials in the present experiments, both reducing and non reducing sugars were assayed in relation to soaking injury. (Sugars were assayed because they form a common link, as the starting point of glycolysis so that seeds with differing storage materials may readily be compared.)

Calculation of the loss of sucrose (mg g dry weight^{-1} hr^{-1}) from seeds on soaking (calculated from the fitted regression lines for the fall in sucrose with duration of soaking) showed a very close positive correlation against QCO_2^n ($p=.001$) (Table 53 and Figure 41).

Similarly, Kerr (1964) found a greater loss of sugars from seeds as a result of increasing soil water while Mathews and Carver (1971) found that soluble carbohydrate exudate into soakwater gave a good indication of field emergence in peas. Brown and Kennedy (1966) working with soy bean seeds showed that exudation from the seed increased

with low oxygen availability.

This fall in sugars has been related to increased susceptibility to pathogenic attack (Heydecker, 1968); and tests on electrical conductivity of soakwater (Mathews and Carver, 1971) probably monitor the internal changes taking place within the seed.

Indeed, when anaerobic respiration is high, as in seeds intolerant of soaking injury, metabolism of the reserves within the seed will also be rapid and the carbohydrate reserves are likely to become depleted more rapidly. This will be especially apparent if the utilization of reserves is relatively inefficient, as in this case when a substrate is respired anaerobically, when for a given amount of energy disproportionately higher respiration rates are necessary.

The main loss of sucrose is probably in the general metabolism of the seed in a bid to provide energy for the seed, and contributory to the loss is general leakage from the seed, therefore in seeds with a high QCO_2^n earlier cessation of growth as a result of soaking injury is likely to occur (ie. when the reserves have been completely utilized).

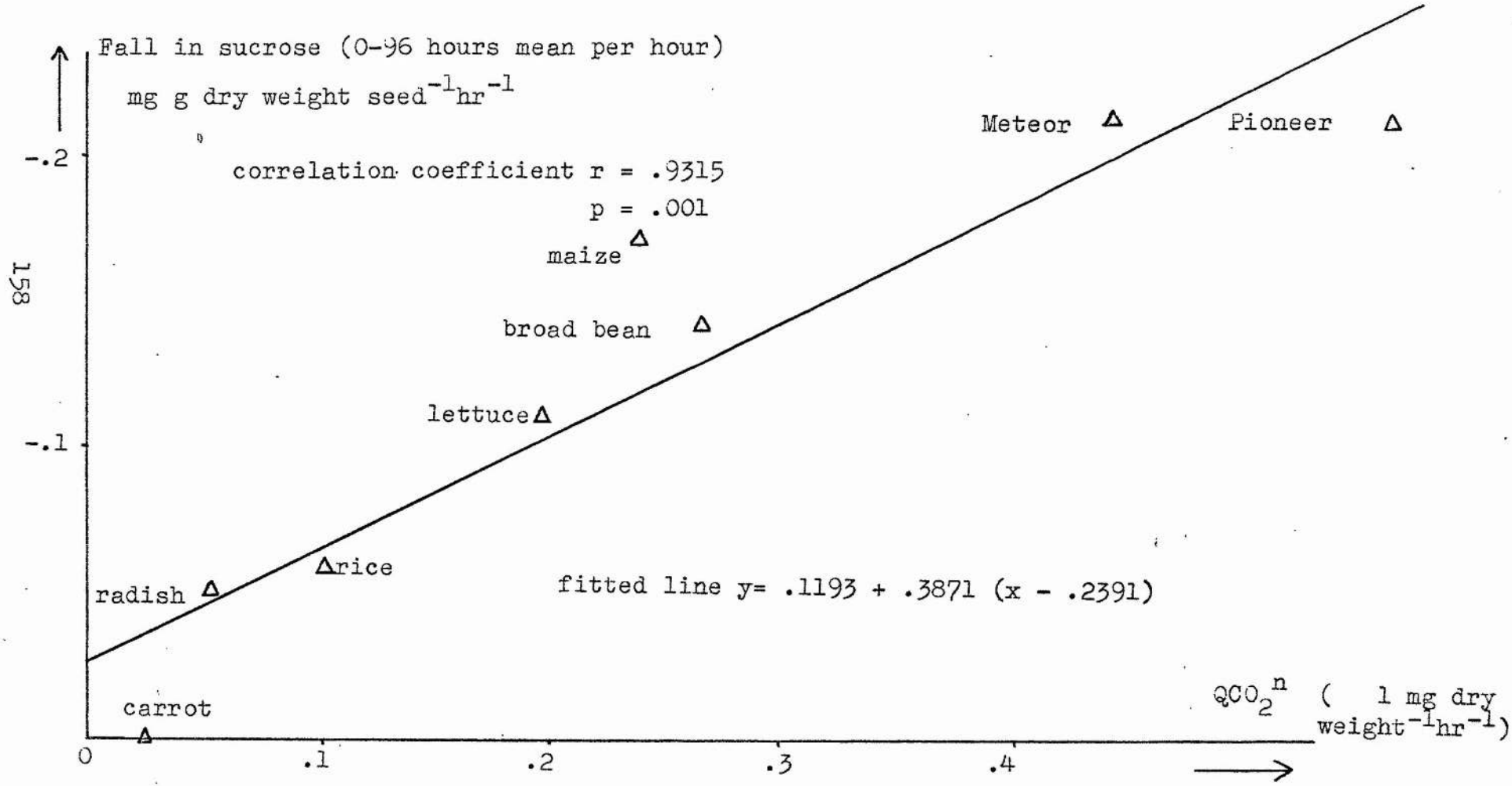
Table 53. Loss of sucrose (mg g dry weight seed^{-1} hr^{-1}) from seeds on soaking (calculated from fitted regression lines for fall in sucrose with duration of presoaking treatment for 0 to 96 hours) and its relationship to respiration under nitrogen after a 24 hour presoaking treatment (μ l mg dry weight^{-1} hour^{-1}) in a range of crop species.

Species or cultivar	Loss of sucrose (mg g dry weight seed^{-1} hour^{-1})	Respiration under nitrogen (μ l mg dry weight^{-1}hr^{-1})
Meteor pea	-.2134	.445
Pioneer pea	-.2103	.569
maize	-.1699	.240
broad bean	-.1435	.269
lettuce	-.1080	.199
rice	-.05767	.103
radish	-.05154	.0616
carrot	-.001370	.0263

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	.03707	1	.03707	39.115
Residual	.005658	6	.0009430	
Total	.04273	7		p=.001

Figure 41. Fitted regression line for fall in sucrose (mg g dry weight seed^{-1} hr^{-1}) against respiration under nitrogen (μl mg dry weight seed^{-1}hour^{-1}).



Anaerobiosis and fermentation - Changes in ethanol content

It has therefore been shown that when seeds are soaked underwater, those with the highest respiration rate under nitrogen consume their seed reserves more rapidly, and they are also the seeds with the poorest survival ability underwater. Since underwater germination is largely anaerobic it is then logical to consider the anaerobic breakdown of sugars in previously recorded systems.

Under complete anoxia respiratory metabolism from sugar to pyruvate will occur (ie. glycolysis) but further oxidation via the tricarboxylic acid cycle (TCA cycle) will be inhibited. Normally pyruvate would accumulate in excess because it cannot be oxidised under anoxia (oxygen is normally required at the terminal oxidase systems), but its fate will be determined by the metabolic equipment possessed by the organism, which may be able to dispose of pyruvate by reduction and this will in turn determine the soaking tolerance of the seed; ie. if respiration is blocked then metabolic poisoning will occur at the point of the block and further metabolism will be inhibited, but if the organism is able to convert pyruvate to a non toxic end product then it will increase its survival chances. However, alcohol fermentation will normally

commence in plant tissues in the presence of pyruvic decarboxylase and alcohol dehydrogenase which decarboxylate pyruvate and reduce acetaldehyde respectively to form alcohol (James, 1971 and 1953). This reaction is extraordinarily complete in many species eg. in carrot where the reaction goes almost to completion and satisfies the Gay Lussac equation to within one per cent (James, 1953). Thus in the reduction from sugar to alcohol, the overall reaction may be expressed by

$$C_6H_{12}O_6 \rightleftharpoons 2C_2H_5OH + 2CO_2$$

This reaction is typified by the equimolar production of alcohol and carbon dioxide (eg. Hatch and Turner, 1958 in pea seed extract), indeed several authors have used this criterion as confirmation of anaerobic respiration in that a greater production of carbon dioxide indicates aerobic or at least a mixture of aerobic and anaerobic respiration where

$$C_6H_{12}O_6 + 6O_2 \rightleftharpoons 6CO_2 + 6H_2O$$

Alcohol fermentation occurs during the normal germination of many seeds which undergo a certain amount of anaerobiosis due to the impermeability of the seed coat to oxygen (Spragg and Yemm, 1959). There is much evidence for the accumulation of alcohol up to the point of rupturing the seed

coat ie.radicle emergence (eg. Cossins and Turner, 1962).

In the present experiments alcohol in seeds increased on soaking for 96 hours (compared with controls) in all species except lettuce (Tables 21 and 22). This is beyond the duration of natural anaerobiosis due to impermeability of the seed coat eg. 5 to 6 hours in Phaseolus mungo (Morohashi and Shimokoriyama, 1972), 30 minutes in Sinapsis alba (Spedding and Wilson, 1968), 1 hour in Lactuca sativa (Haber and Tolbert, 1959) thereafter aerobic respiration will commence, at least in part.

In the soaking tolerant species used in the present experiments the rise of ethanol was smaller (0.3 to 3.3 times on soaking for 96 hours compared with control) than intolerant species where a greater rise in ethanol was recorded on soaking (3.8 to 17.0 times). Tyler (1969) and Crawford and Tyler (1970) found a similar disproportionate rise in ethanol on flooding the roots of several species of grown plants which were intolerant of experimental flooding. They did not find such a large increase in species tolerant of this treatment. This rise is indicative of a much higher metabolic rate and they concluded that there

was an acceleration of glycolysis as a result of a reversal of the Pasteur effect in flood susceptible species. The parallel rise for seeds intolerant of soaking injury reported here is leant credence by the high respiration rates under nitrogen of seeds susceptible to soaking injury.

Alcohol accumulation has been shown to depend upon whether seeds are germinated aerobically or anaerobically (eg. Cossins and Beevers, 1963; Cossins and Turner, 1963; Cossins, 1962) as in the case of underwater germination alcohol increased in some species (Doireau, 1969). Accumulation of alcohol may be sustained under anaerobiosis eg. in Phaseolus active accumulation occured for 6 days (Sherwin and Simon, 1969). It is likely, therefore, that the duration of soaking treatment will determine the extent of injury in seeds as a result of alcohol accumulation. However, when regression analyses were carried out for ethanol against duration of presoaking treatment (Table 69-73 appendix) in each species, no significant linear regressions were given. This does not mean that ethanol does not accumulate in a linear fashion, but in these experiments changes within the seed were examined rather than the total change which would include some diffusion of ethanol into

the soakwater. There was probably some loss in this way, which may be reflected in the non linearity of the analyses. Also the reaction is likely to slow with time due to the depletion of reserves and to product or substrate inhibition where there is a high rate of respiration.

However, when the ratio of the increase of ethanol after soaking for 96 hours (ratio of ethanol after 96 hours soaking to ethanol after 96 hours germination on moist filter paper) was compared with the species respiration rate under nitrogen, a significant positive correlation was found ($p=.05$)(Table 54 and Figure 42). This confirms that those species with the highest respiration rates under nitrogen, were increasing their alcohol content more rapidly, thus resulting in earlier damage to the seed. This presupposes the availability of sugar reserves within the seed, so that when the rate of alcohol increase was compared with sucrose loss from the seed per hour a significant positive correlation was again found ($p=.05$)(Table 55 and Figure 43) which confirms the high rate of sugar consumption.

This is further evidence for an active alcohol fermentation at the expense of reserves within the seed. James and Ritchie (1954) found a similar relationship between the sugar used by carrots

under anaerobiosis and alcohol fermentation. They also related this to the equimolar production of carbon dioxide, but in the present experiments such a relationship could not be demonstrated because carbon dioxide emission and alcohol accumulation were not measured in the same reaction mixtures at the same time.

Damage to the seed would then result from ethanol damage to the meristems and the unavailabilty of reserves for developmental metabolism in the seed because of the energy loss in sugar conversion to alcohol. Also alcohol can only be reconverted slowly and with difficulty to assimilatory products. Such a conversion, via the glyoxylate cycle, has been reported in many species when aerobic conditions resume (eg. Peterson and Cossins, 1966 in castor bean), however after a sustained period of anaerobiosis it is unlikely that such a conversion could be rapid enough to consume all the ethanol produced.

Table 54. Regression analysis for the ratio of ethanol in seeds soaked for 96 hours underwater to controls germinated for 96 hours on moist filter paper against respiration under nitrogen after germina tion for 24 hours underwater (μ l mg dry weight seed^{-1}hour^{-1}) in the seeds of several crop species.

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	42.1640	1	42.1640	6.9271
Residual	36.5210	6	6.0868	
Total	78.6850	7		p=.05

Table 55. Regression analysis for the ratio of ethanol in seeds soaked for 96 hours underwater to controls germinated for 96 hours on moist filter paper against fall in sucrose per hour (calculated from the regression equation for mg sucrose g dry weight seed^{-1} against duration of presoaking treatment for each species) in a range of crop species.

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	122.0783	1	122.0783	10.9175
Residual	89.4551	8	11.1819	
Total	211.5334	9		p=.025

Figure 42. Fitted regression line for ratio of increase in alcohol on soaking (for 96 hours germination underwater to 96 hours germination on moist filter paper) against respiration under nitrogen (μl mg dry weight seed^{-1}hour^{-1}) in the seeds of several crop species.

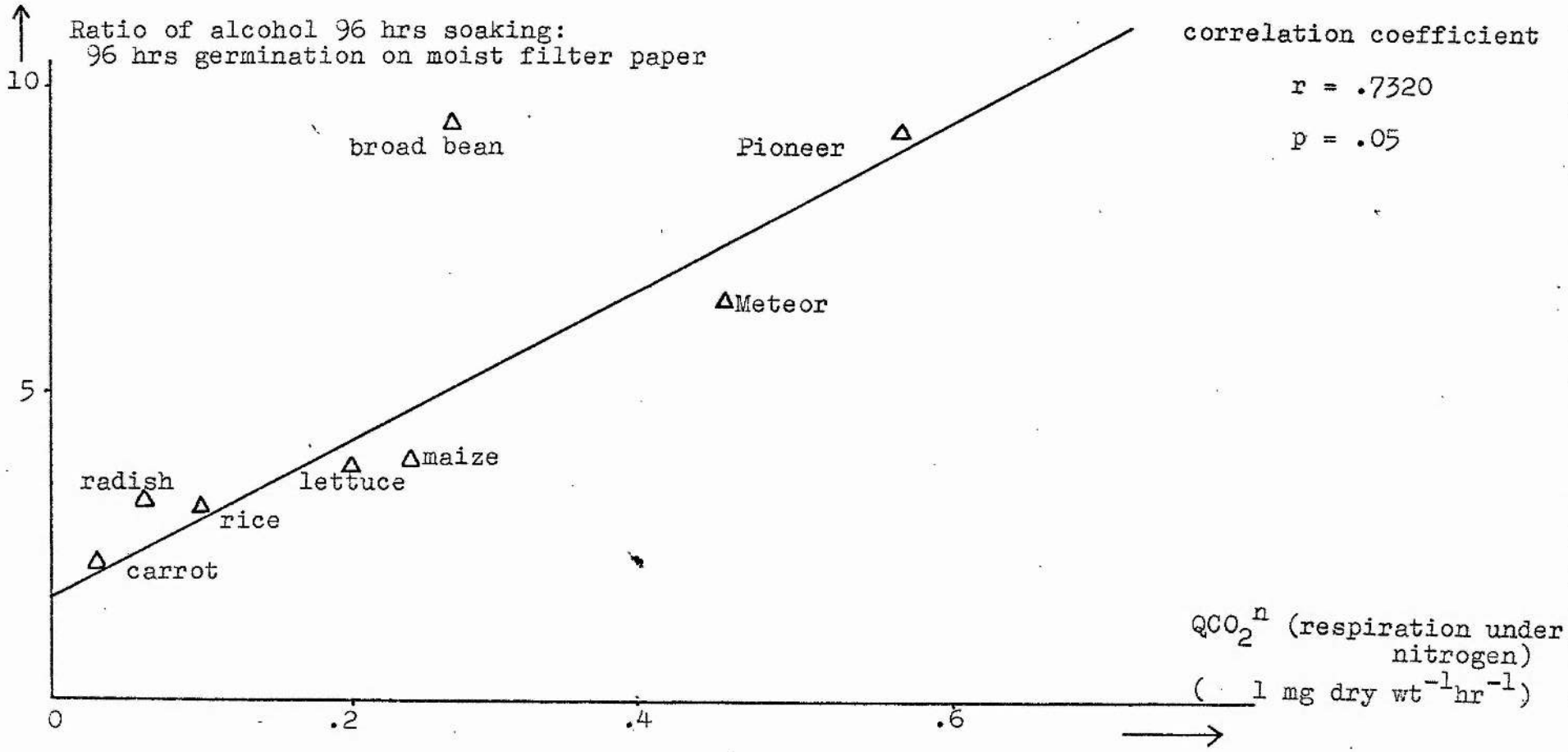
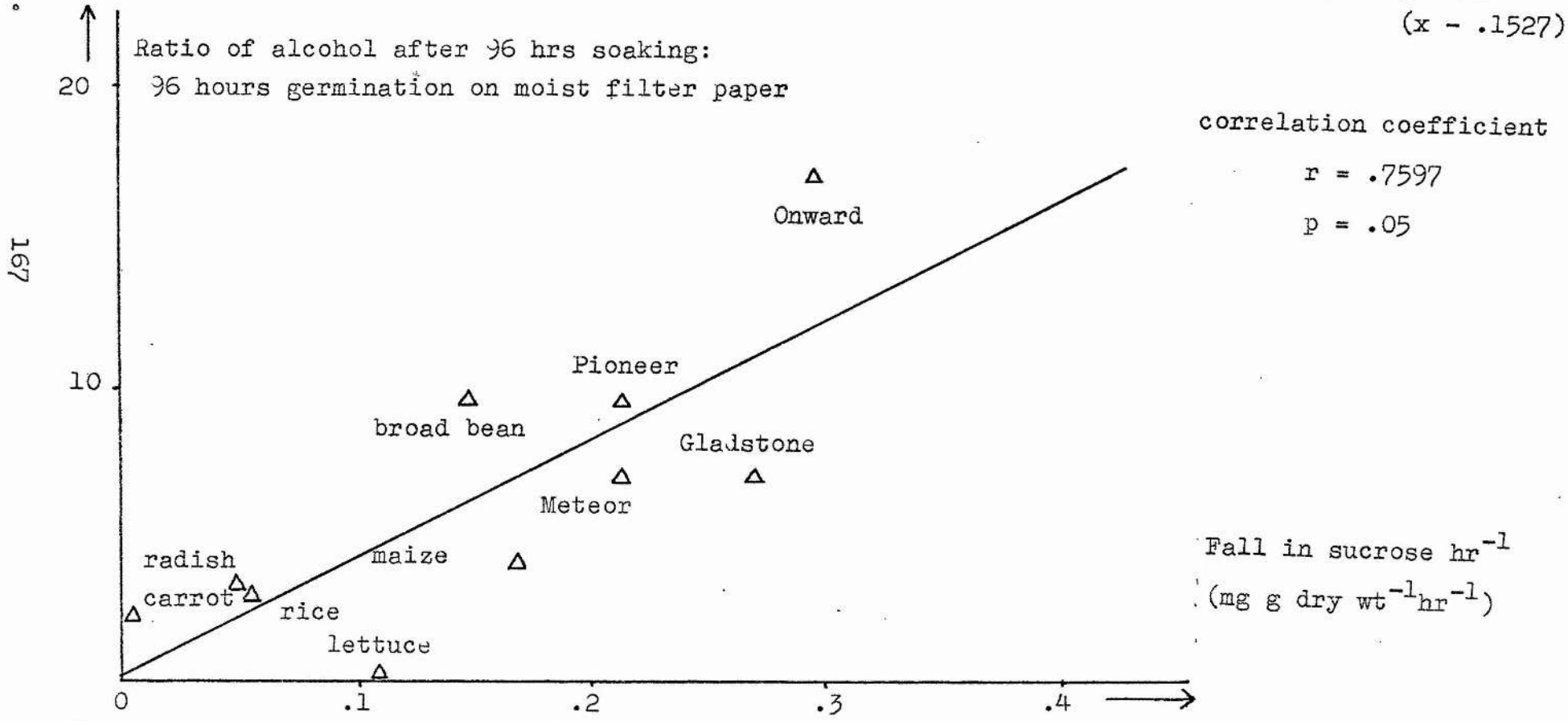


Figure 43. Fitted regression line for ratio of increase in alcohol on soaking (for 96 hours germination underwater to 96 hours germination on moist filter paper against fall in sucrose per hour (mg g dry weight seed^{-1}hour^{-1}) in the seeds of several crop species.



fitted line

$y = 6.2054 + 38.2194 (x - .1527)$

correlation coefficient

$r = .7597$

$p = .05$

Changes in malic acid content of seeds on soaking

Accumulation of alcohol is the most frequently reported end product of anaerobiosis in plants, but many other end products exist. Malic acid has also been identified in this context (Crawford and Tyler, 1969). In the present thesis malic acid assays were conducted in relation to duration of soaking treatment.

On soaking seeds malic acid showed no significant trend with duration of soaking treatment, but the increase in malic acid ratio (ratio of malic acid after 96 hours soaking to malic acid content of seeds after germination for 96 hours on moist filter paper) was negatively correlated with anaerobic respiration rate ($p=.001$)(Table 56 and Figure 44). Thus in species tolerant of soaking, malic acid content of the seeds increased as the respiration rate under nitrogen fell, while malic acid content was negatively correlated with loss of sucrose from the seed ($p=.001$)(Table 57 and Figure 45).

When increase in malate ratio was compared with increase in ethanol ratio a significant inverse relationship was again given ($p=.02$)(Table 58 and Figure 46). Thus as alcohol increased so malic acid fell in such a way that the fitted regression

line appeared to be in the form of a cluster analysis with species intolerant of soaking grouped together, and those tolerant of soaking grouped separately together (Figure 46). Again, this is evidence for different behavioural classes with respect to germination mirroring their metabolic behaviour.

This is in accordance with the work of Crawford and Tyler (1970) who found an increase in malic acid in species tolerant of experimental flooding and an increase in alcohol in species intolerant of this treatment. Therefore malate to ethanol ratios gave higher values for species tolerant of flooding in their experiments. ie. there was an acceleration of glycolysis in the intolerant species with ethanol production, while tolerant species did not show an acceleration of glycolysis to the same degree but accumulated malic acid in preference.

A metabolic scheme was proposed by McManmon and Crawford (1970) with a differential shift to malate in flood tolerant species. They found that in a range of plants, that those species with higher malate levels after flooding were more tolerant of this treatment, while those intolerant of flooding accumulated more alcohol. In the intolerant group 'malic enzyme' was demonstrated while its presence was not shown in the tolerant group (Figures 47 and 48).

Table 56. The relationship between respiration under nitrogen (μ l mg dry weight $seed^{-1}hour^{-1}$ after 24 hour soaking treatment) and increase in malic acid after germination for 96 hours underwater compared with controls germinated for 96 hours on moist filter paper (expressed as ratio underwater: control) in the seeds of several crop species.

Species or cultivar	Respiration under N_2 (μl mg dry $wt^{-1}hr^{-1}$) at 24 hours	Malic acid ratio (96 hrs germination underwater:96hr control)
Pioneer pea	.569	.07323
Meteor pea	.445	.2761
broad bean	.269	1.138
maize	.240	1.037
lettuce	.199	1.150
rice	.103	1.379
radish	.0616	1.353
carrot	.0263	1.430

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	1.7619	1	1.7619	92.8539
Residual	.1138	6	.01898	
Total	1.8758	7		p=.001

Figure 44. Fitted regression line for increase in malic acid on soaking (ratio of malic acid after 96 hours germination underwater to malic acid after 96 hours germination on moist filter paper) against respiration under nitrogen (μ 1 mg dry weight seed^{-1} hour^{-1} at 24 hours soaking) in seeds of several crop species.

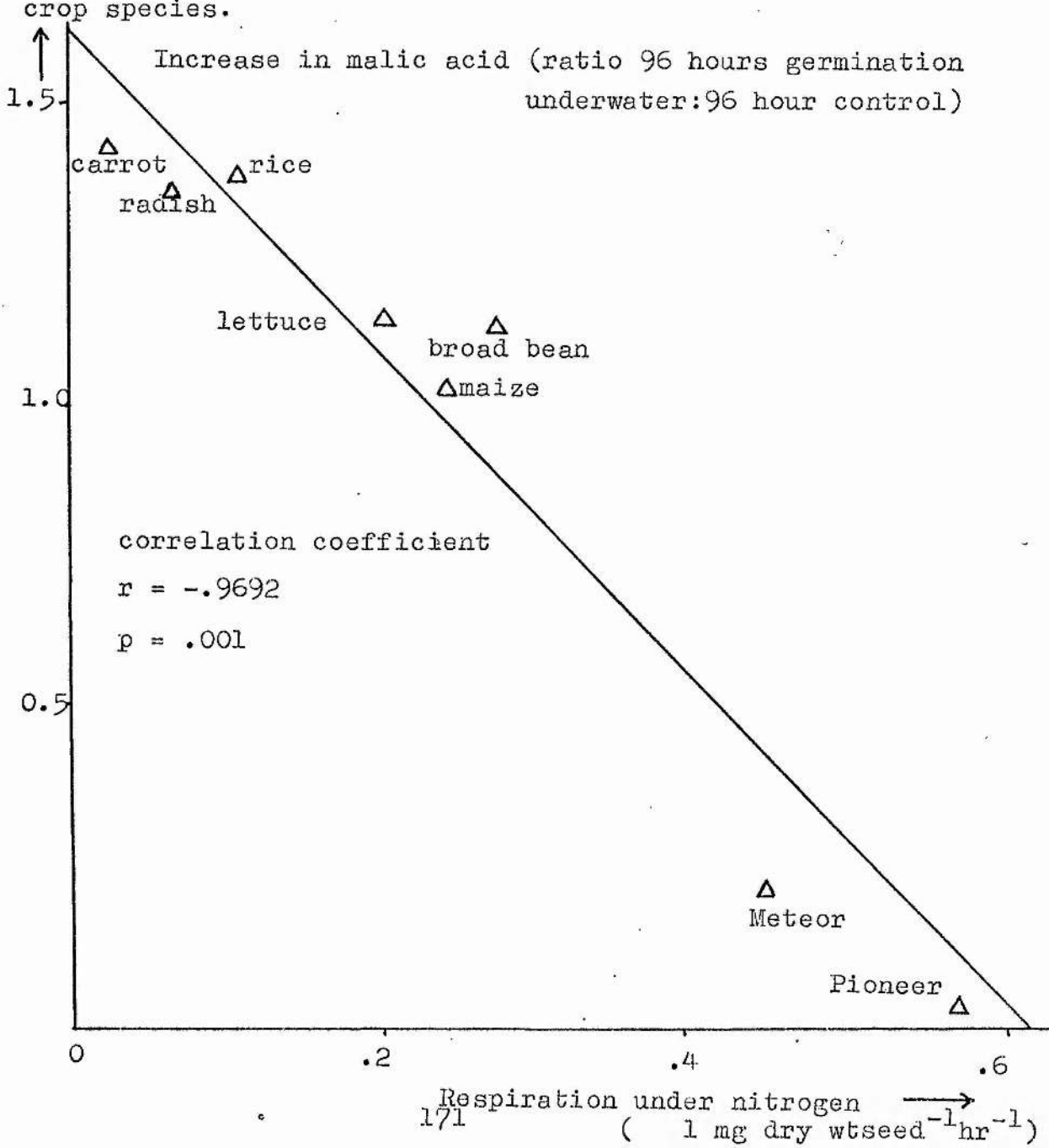


Table 57. Regression analysis for change in malic acid in seeds of several crop species after soaking for 96 hours underwater (ratio of 96 hours underwater to 96 hours germinated on moist filter paper) against fall in sucrose per hour (mg g dry weight $seed^{-1}hr^{-1}$).

Species or cultivar	Ratio of malic acid after 96 hrs under water:96 hr control	Fall in sucrose hr^{-1} (mg g dry weight $seed^{-1}hour^{-1}$)
Gladstone pea	.02134	-.2639
Pioneer pea	.07323	-.2103
Onward pea	.1603	-.2931
Meteor pea	.2761	-.2134
broad bean	1.138	-.1435
maize	1.037	-.1699
lettuce	1.150	-.1080
rice	1.379	-.05767
radish	1.353	-.05154
carrot	1.430	-.001370

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	2.6565	1	2.6565	43.1521
Residual	.4925	8	.06156	
Total	3.1490	9		p=.001

Figure 45. Fitted regression line for increase in malic acid in the seed of several crop species on soaking for 96 hours (ratio of malic acid after germination for 96 hours underwater to 96 hour control germinated on moist filter paper) against fall in sucrose per hour (mg g dry weight seed^{-1}hr^{-1}).

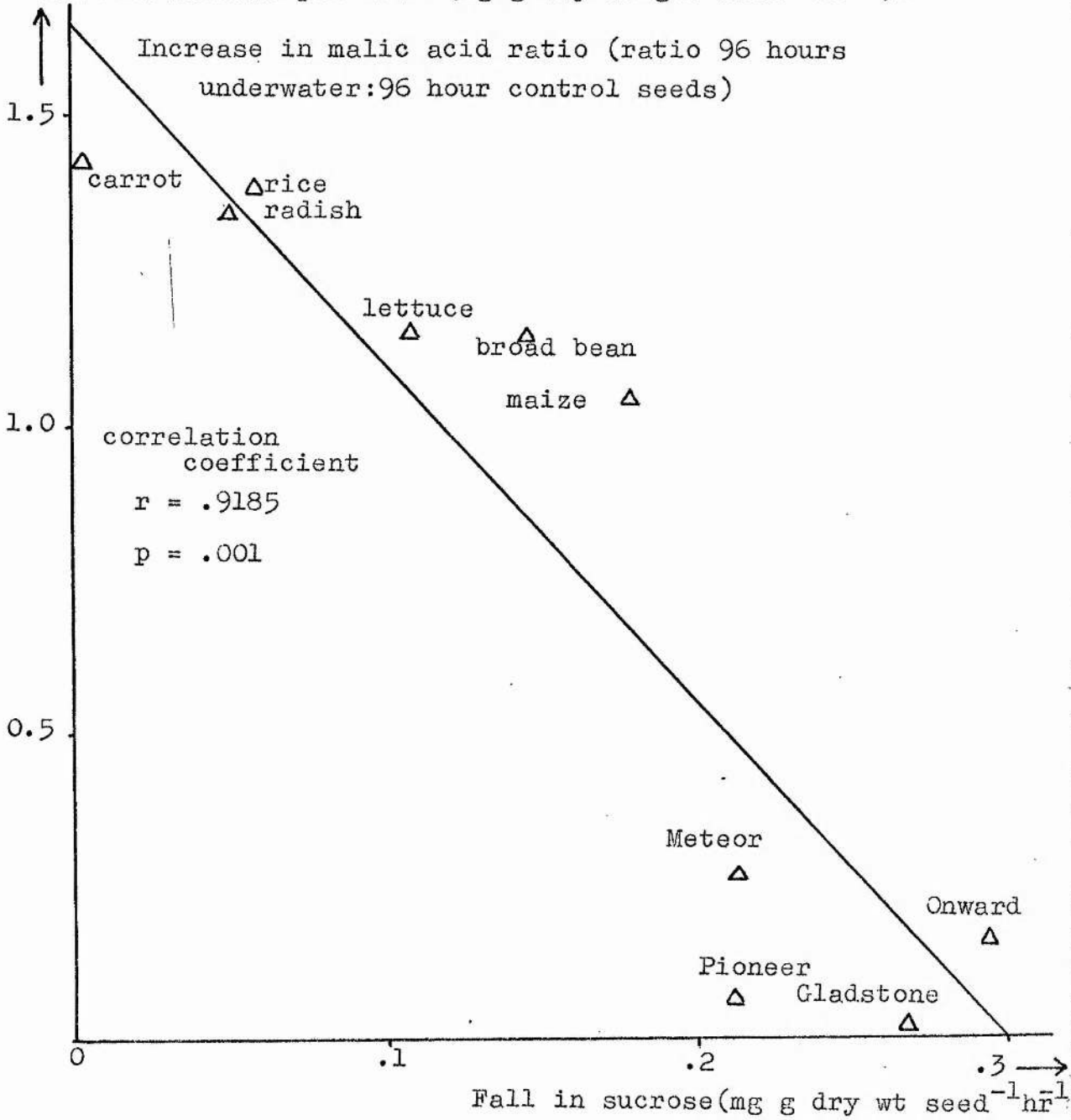


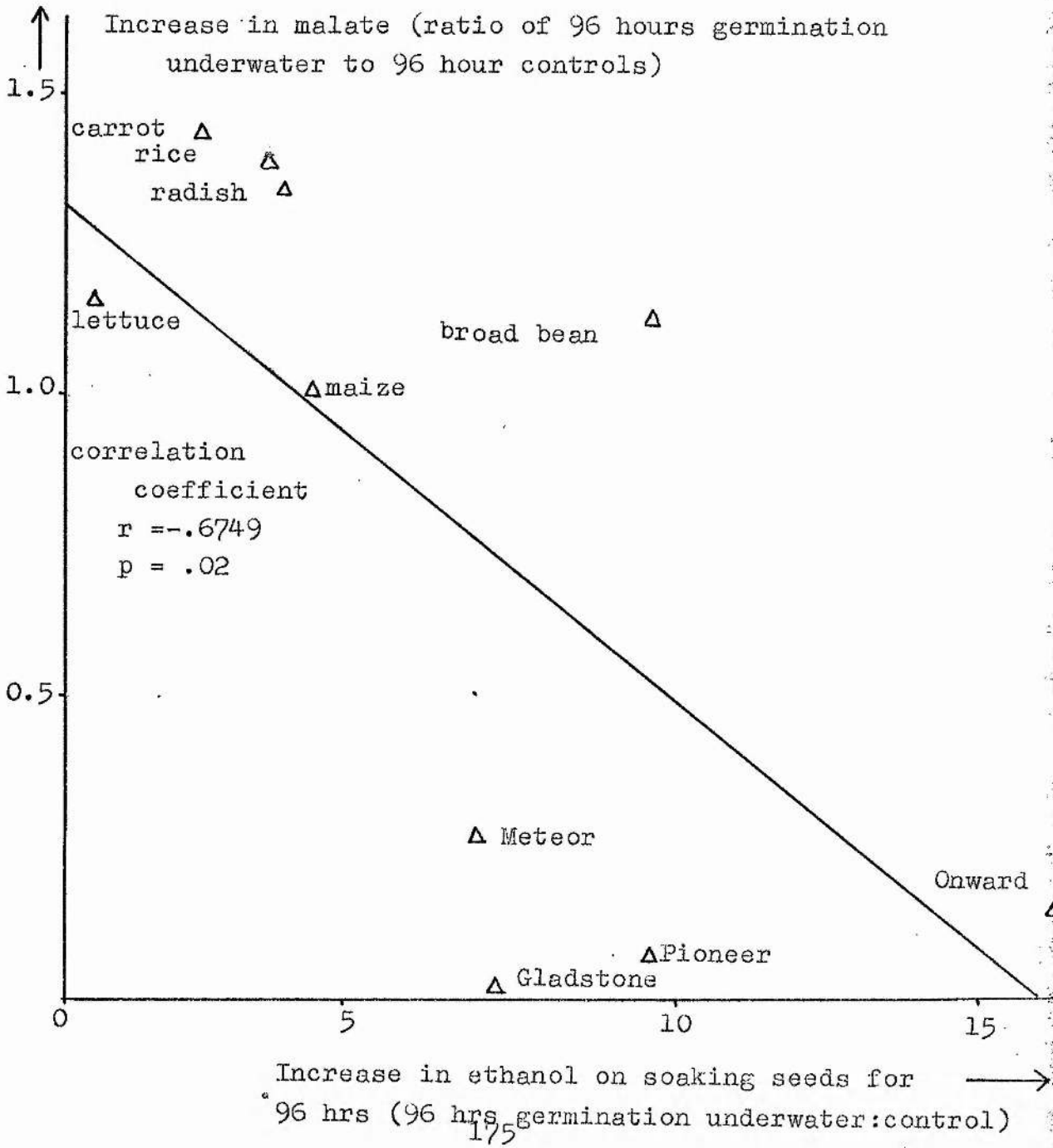
Table 58. The relationship between the increase in alcohol and in malate on soaking seeds of several crop species (both expressed as the ratio of the value for seeds germinated for 96 hours underwater to the value for control seeds germinated for 96 hours on moist filter paper).

Species or cultivar	Ratio of 96 hours germination underwater: 96 hour control on moist filter paper. Alcohol increase.	Malic acid increase.
Onward pea	16.9598	.1603
broad bean	9.5175	1.1376
Pioneer pea	9.2999	.07323
Gladstone pea	6.9804	.02134
Meteor pea	6.6056	.2761
maize	3.8115	1.0370
radish	3.3283	1.3534
rice	3.0390	1.3793
carrot	2.1444	1.4300
lettuce	.3689	1.1503

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	1.4345	1	1.4345	6.6908
Residual	1.7152	8	.2144	
Total	3.1497	9		p=.05

Figure 46. Fitted regression line for change in malate on soaking seeds for 96 hours (ratio of malate for 96 hours germination underwater to 96 hour controls germinated on moist filter paper) against change in alcohol on soaking in several crop species.



The importance of malic acid is therefore as an alternative non toxic end product to alcohol.

McManmon and Crawford based their theory on the absence of malic enzyme in flood tolerant species. However, Davis, Nascimento and Patil (1974) using the same flood tolerant species demonstrated the presence of malic enzyme in all of these species. There is obviously some disparity between the results of McManmon and Crawford and those of Davis etal but it should be emphasised that McManmon and Crawford did find a difference in malic enzyme activity between flood tolerant and flood intolerant species. It may therefore be argued that their theory still holds good and that it is possible that this difference is quantitative or indeed that there may be some difference in activity of the enzyme from flood tolerant plants for example due to the presence of an inhibitor.

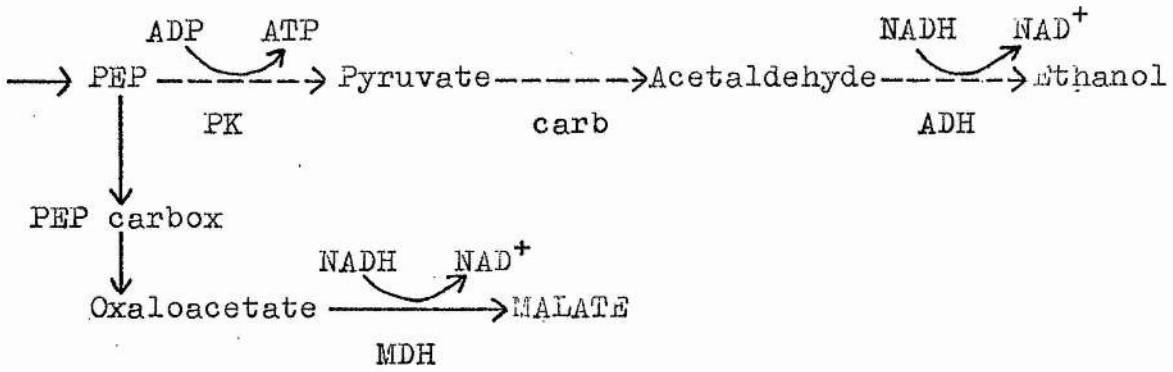
As the discussion of malic acid accumulation is relevant to this thesis it is considered prudent to compare the difference found between McManmon and Crwford's paper and that of Davis etal in more detail. Davis etal demonstrated activity in the 'tolerant group' using a different extraction method which was shorter and of more recent origin. However, comparing extraction techniques is

difficult but of the several possible differences perhaps the most important is the addition of Polyclar by Davis etal. This is added to absorb phenolic materials which are often released on grinding plant material and may be inhibitory to enzyme activity. There are many references to phenol inhibition of germination eg. in lettuce seed phenols may interfere with the normal functioning and respiratory metabolism (Mayer, 1963). There was no similar additive to absorb phenolic compounds in McManmon and Crawford's assay (taken from Ochoa, 1955).

That this hypothesis may be true is supported by Davis etal in the conclusion to their paper, where the existence of an inhibitor of malic enzyme was reported in potato tubers. This inhibitor could be a phenolic compound. If this inhibitor, whatever it may be, is present in flood intolerant plants it could account for the lack of malic enzyme activity observed by McManmon and Crawford in these plants; and its presence would be a determinant of soaking tolerance.

Figure 47.

Metabolic scheme for flooding tolerant species (after McManmon and Crawford, 1970).



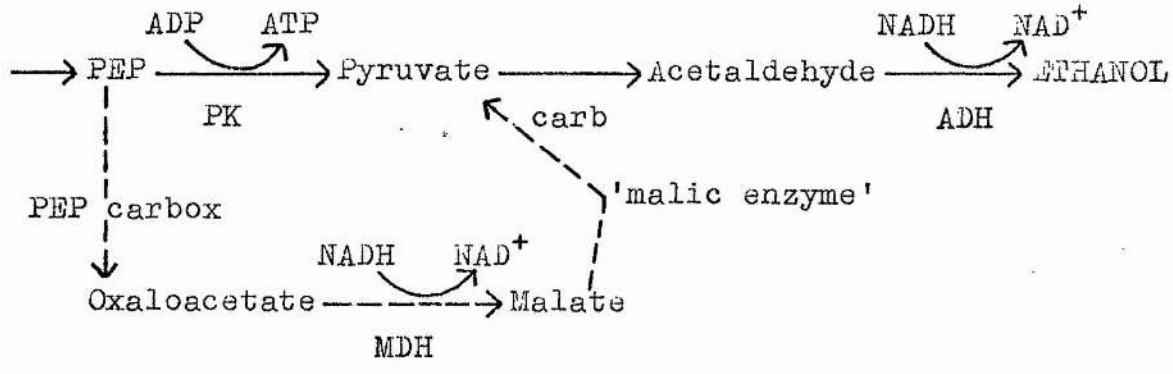
PK = pyruvate kinase

PEP carbox = phosphoenolpyruvate carboxylase

carb = carboxylase.

Figure 48.

Metabolic scheme for flood intolerant species (after McManmon and Crawford, 1970).



PK = pyruvate kinase carb = carboxylase

PEP carbox = phosphoenolpyruvatecarboxylase

Changes in lactic acid on soaking seeds

Lactic acid is often formed under anaerobiosis (eg.Wager, 1961 in peas; Sherwin and Simon, 1969 in beans) and it may also occur under flooding (Hook, Brown and Kormanik, 1971 in swamp tupelo), indeed this is almost the universal answer to anoxia in the animal kingdom. However, its occurrence in plants is much less frequent or at least at much lower concentrations. There are several possible explanations for this but perhaps the most important is the possession of the enzyme pyruvic decarboxylase. This enzyme will secure pyruvic acid even when lactic dehydrogenase is present and extracts the reaction

$$CH_3COCOOH \underset{\text{pyruvic decarboxylase}}{\rightleftharpoons} CH_3CHO + CO_2$$

which goes virtually to completion. Thus lactate does not occur in tissues possesing this enzyme.

In the present experiments there was no real evidence for a linear increase in lactic acid with duration of soaking treatment (Table 77 appendix), nor was a significant relationship demonstrated for the ratio of lactic acid content of seeds soaked for 96 hours to lactic acid content in seeds germinated for 96 hours on moist filter paper against respiration under nitrogen. (Table 80 Appendix).

When lactic acid was expressed as the percentage of the total anaerobic end products measured per g dry weight seed (μ M ethanol + μ M malate + μ M lactate g seed^{-1}) then a significant negative correlation between respiration under nitrogen and percentage lactic acid was found in the soaking (96 hour) treatments using a Kendall Rank test ($p=.007$)(Table 59). No such correlation was found for control seeds germinated on moist filter paper.

Therefore, although lactic acid accumulation is not directly related to absolute respiration rate under nitrogen it does form a higher proportion of the anaerobic product in seeds where the QCO_2^n is lower ie. soaking tolerant seeds where the total anaerobic product is also smaller.

This shift in favour of lactate is important because it is possible to repay the oxygen debt via the reverse reaction

$$CH_3CHOHCOOH + NAD^+ \longrightarrow CH_3COCOOH + NADH$$

lactic acid LDH pyruvic acid.

At the same time lactic acid is a non toxic product and can accumulate within the seed without deleterious effects.

Analysis for lactic acid content of seeds can be used to monitor the presence of the pyruvic to lactic acid reaction and thus the absence of the

Table 59.

Lactic acid content of seeds expressed as a percentage of total anaerobic end products assayed (μ M ethanol + μ M malate + μ M lactate g seed^{-1}) with Kendall Rank test for percentage lactic acid against respiration under nitrogen (μ l mg dry weight seed^{-1} hour^{-1}).

Species or cultivar	Percentage lactate 96 hours underwater	96 hour control	Anaerobic respiration QCO_2^n 24 hours soak (μ l mg dry wt^{-1}hr^{-1})
Pioneer pea	.60	2.29	.569
Meteor pea	1.04	6.83	.445
broad bean	1.36	38.37	.269
maize	3.46	11.20	.240
lettuce	44.17	16.77	.199
rice	9.68	3.12	.103
radish	25.76	12.14	.0616
carrot	24.49	37.10	.0263

Kendall Rank Correlation Coefficient

S	= -20	= -10
probability		
p	= .007	= .138 (N.S.)

enzyme pyruvic decarboxylase (or atleast relative inactivity of this enzyme) which is essential for alcohol formation. For example in potato tissue under nitrogen, carbon dioxide output falls from normal respiration levels (CO_2 is released when pyruvic acid is decarboxylated to acetaldehyde by pyruvic decarboxylase), but on return to air there is a burst of carbon dioxide emission before the rate returns to normal (James, 1953) ie.Potatoes which have been shown to have very little pyruvic decarboxylase activity form no alcohol under nitrogen, but instead a mixture of products forms of which lactate constitutes about half. (the burst of CO_2 emission is due to the oxidation of lactate.) When a similar experiment was conducted with carrot tissue under nitrogen, carbon dioxide output rose, while on return to air the level immediately returned to normal. In carrot tissue alcohol formation occurs under nitrogen and its formation is largely irreversible with no corresponding oxidation on return to air (James, 1953).

Lactic acid has been reported in seeds by several other authors during the period of partial anaerobiosis at the start of germination due to impermeability of the seed coat to oxygen (eg. Zeleneva, 1972; Cossins, 1964). This

impermeability may last for up to thirty hours (Sherwin and Simon, 1969 in Phaseolus vulgaris) while the reverse reaction has been reported under aerobic conditions in seeds (eg. Cossins, 1964).

These changes in lactic acid must be distinguished from changes as a result of enforced anaerobiosis (flooding). Sherwin and Simon (1969) did report such a change in Phaseolus seeds where the content of lactic acid rose with dampness of the sand. They also found an increase in lactic acid content on transferring seeds to an anaerobic environment. However, Tyler (1969) concluded that lactic acid was relatively unimportant as a product of anaerobic respiration under flooded conditions in grown plants, although he found increases in this acid in the roots of several species on experimental flooding of their roots, but these effects were probably not significant.

An important difference has been established therefore, because lactic acid has been shown to be an important diversionary end product of anaerobiosis in seeds. This difference may be due to the absence of pyruvic decarboxylase, or its existence in a relatively inactive form in seeds, especially in soaking tolerant seeds. Where pyruvic decarboxylase is present it converts

Pyruvate to acetaldehyde which is itself an inductive agent for alcohol dehydrogenase (McManmon and Crawford, 1969) which would favour alcohol production at the expense of lactic acid.

Changes in properties of enzymes on soaking seeds

The accumulation of alcohol, malate and lactic acid will depend upon the presence and properties of the enzymes catalysing the reactions involved. To extend the observations of McManmon and Crawford further experiments were carried out on alcohol and malic dehydrogenases with reference to soaking injury in seeds. This affords a comparison with the literature relating to lactic dehydrogenase where the nature of this enzyme and changes under anaerobiosis have been extensively investigated, especially in the animal kingdom.

Changes in alcohol dehydrogenase

In the present work, two species (including two cultivars of Pisum sativum) of different soaking tolerance were examined. Seeds of most species gave high ethanol contents after soaking for 48 hours (Tables 21 and 22) and therefore studies on enzyme activity were concentrated on soaking treatments up to 48 hours. Alcohol dehydrogenase catalyses the final reaction in the accumulation of ethanol and was therefore selected for study. It catalyses the following reaction-

$$\underset{\text{acetaldehyde}}{CH_3CHO} + NADH \underset{\text{alcohol dehydrogenase}}{\rightleftharpoons} \underset{\text{ethanol}}{CH_3CH_2OH} + NAD^+$$

On soaking the specific activity (mg protein^{-1})

as well as total activity (g^{-1}seed) increased with duration of soaking in the two pea cultivars, while in rice activity fell with soaking (Table 29). Furthermore, the activity of ADH in rice was significantly lower than in the two pea cultivars after soaking (Tables 29 and 30).

It will be seen that ADH is more capable of being induced (or activated in some way) in seeds susceptible to soaking injury, or that it exists at higher levels in seeds of poor soaking tolerance (peas) than in seeds of higher soaking tolerance (rice), so that some difference may exist in the nature of the enzymes between species.

Alcohol dehydrogenase is known to increase its activity under anaerobic conditions (Hageman and Flesher, 1960) or on flooding the roots of species susceptible to this treatment (Crawford and McManmon, 1968) however, it is not clear what the actual mechanism for increase is. Under anaerobic conditions the TCA cycle is blocked which leads to the production of pyruvate, and the excess pyruvate may be decarboxylated to acetaldehyde as already described. Acetaldehyde is capable of inducing ADH (App and Meiss, 1958; Crawford and McManmon, 1968) which results in the accumulation of ethanol.

This is simple induction, in this case causing

an increase in the level or activity of an enzyme already present (Table 28 shows the presence of ADH in dry seeds) ie. there is feedback because as the enzyme is induced so glycolysis accelerates to produce more acetaldehyde which induces the production of more enzyme.

The situation is likely to be more complicated than simple enzyme induction, especially as several authors have shown a change in the affinity of an enzyme for its substrate under anaerobiosis. Such a change is indicated by the Michaelis constant for enzyme with respect to its substrate. (Km values indicate how easily an enzyme catalysed reaction will take place, so that for smaller Km values the faster the reaction will be at the available metabolic concentrations).

During soaking rice had a significantly higher Km for ADH with respect to acetaldehyde up to 48 hours of soaking than Pioneer pea (Figure 12). Meteor and Pioneer pea Km's were partly confounded up to 24 hours but Meteor was significantly higher than Pioneer at 48 hours although confounded with rice. Therefore, although there was little change in Km with duration of soaking treatment (apart from a slight rise at 12 hours soaking in each species) there was a distinct species difference, with higher values recorded in the more tolerant

species ie. a lower Km favours the reduction of acetaldehyde to alcohol (as in species intolerant of soaking where higher Km's were recorded).

A change in Km for ADH with respect to acetaldehyde was demonstrated by McManmon (1969) where a fall in the apparent Michaelis constant was recorded on flooding non helophytes, so that ethanol accumulated more rapidly in these species (Crawford and Tyler, 1969). As distinct from McManmon's work, no change in Km was recorded on soaking seeds, but species difference was present which again suggests a difference in metabolic behaviour of seeds as compared with grown plants.

The nature of the difference between soaking tolerant and susceptible species then appears to be that the apparent Km is lower in seeds susceptible to soaking injury leading to a high metabolic rate resulting in alcohol production. At the same time feedback (as above) will accelerate the reaction leading to the observed reversal of the Pasteur effect. Thus a change in Km or a difference in Km due to species difference involving the last reaction in a chain, will cause a shift in the equilibria throughout and the whole process will be accelerated.

McManmon indicates a further possibility, which is the induction of isoenzymes or specific

subunits of a multiple enzyme form. The term isoenzyme is relatively recent (Markert and Moller, 1959) and their widespread occurrence has only recently been appreciated, thus Scandalios (1974) states 'isoenzymes are the rule rather than the exception as previously believed'. The importance of isoenzymes as regulators of cell metabolism is not yet fully understood.

Nielsen and Scandalios (1971) using maize seedlings grown under anaerobiosis, were not able to detect any new isoenzymes and they concluded that the apparent increase in ADH activity under anaerobiosis was not due to activation or induction of new enzymes. However, they found that one of the enzyme forms, referred to as ADH-1 (specified by a particular gene known as Adh_1) increased significantly under anaerobic conditions as compared with other ADH isoenzymes. This was particularly important because ADH-1 favours the backward reaction from acetaldehyde to ethanol. This effect may be unusual and the role of isoenzymes has not yet been universally proven in this connection.

The most significant difference between seeds of differing soaking tolerance is likely to be an increase, or inherently higher levels, of one of the isoenzyme forms with a lower Km for ADH with respect to acetaldehyde in soaking intolerant species.

It is possible that this isoenzyme could be ADH-1 in species which are intolerant of soaking injury rather than the induction of a completely new isoenzyme. (This is not necessarily the case, because closely related isoenzymes are likely to have similar Km values eg. McManmon (1969) using Carex arenaria found two bands close together on flooding, while normally only one band was present but this was in the same place as the two bands).

Because ADH will catalyse the reaction acetaldehyde $\rightleftharpoons$ ethanol either way, the possibility exists, that a given isoenzyme may favour the reaction more strongly in one direction, which in the case of a shift to the right (ethanol) would be a partial explanation of the differing behaviour of ADH under anoxia (eg. ADH-1 favours the reaction to the right).

During the germination of seeds, alcohol does not normally accumulate, although there are periods of anaerobiosis when it is produced quite rapidly (eg. App and Meiss, 1958) and, as already discussed, remetabolism via the glyoxylate cycle may be only part of the picture. It is known that activity of ADH decreases with germination and it has been suggested (Scandalios, 1974) that an inhibitory substance may be produced in the later stages of germination ie. inhibitory to ADH. A further

possibility exists therefore, that this inhibitor may not be produced on soaking seeds of susceptible species, but this is purely speculative as the inhibitor has not yet been identified, although Scandalios claims that the inhibition is irreversible and that the inhibitor itself is heat labile and partly dialyazible. There are reports in the literature of ADH induction by long chain fatty acids (Erikson, 1967; Kollofell, 1970), alternatively oxygen would act as an effective inhibitor of ADH activity.

Changes in malic dehydrogenase activity on soaking seeds

When malic dehydrogenase activity was examined in seeds, the specific activity (mg protein^{-1}) was very much higher in rice seeds up to 24 hours soaking than in pea seeds, although at 48 hours all three species were confounded. There was therefore much higher MDH activity in the early stages of soaking in the tolerant species. However, if the change in MDH activity (comparing unsoaked seeds with seeds soaked for 48 hours) was compared then the specific activity per mg protein rose with duration of soaking in peas ($p=.02$) but not in rice (Table 33). Indeed, McManmon (1969) demonstrated increases in MDH under severe anoxia in helophytes only, but concluded that the precise

role of MDH in flooding tolerance was obscure as he found that both malate and oxaloacetate were inductive agehts, but only at much higher concentrations than might naturally be found ~~naturally~~ under anaerobiosis.

The change in activity per g seed (Table 34) gave similar results, but the relative orders of magnitude were reversed. In rice a lower total activity was recorded which suggests that rice MDH may be a higher protein type or perhaps a different isoenzyme. To investigate these differences further, Km's with respect to oxaloacetate were examined.

The Km for MDH with respect to oxaloacetate rose on soaking in rice, but there was no change in pea (Table 35) and this mirrors the fall in specific and total activities after 48 hours soaking seed of rice (presumable metabolism slows under prolonged anoxia). McManmon suggests that the fall in malic acid observed when non helophyte roots were flooded was by conversion of the malate to other metabolites via oxaloacetate. It is likely that in rice where Km rose, that such a reconversion did not occur thus accounting for the observed accumulation of malic acid.

A significantly lower Km was recorded in rice than in the pea cultivars after 48 hours soaking

which corresponds with the high malate production in this species under anaerobiosis.

Isoenzymes for MDH have been reported (eg. McManmon, 1969) although their role under anaerobiosis has not been elucidated. It is unlikely that the patterm will be directly comparable with ADH.

Changes in lactic dehydrogenase (not investigated)

Recent work on the enzyme lactic dehydrogenase and lactic acid accumulation throws light on the accumulation of lactate as an end product of anaerobiosis. Also a comparison may show similarities with ADH activity.

Two major form of LDH exist-

1. H type which is common in aerobic cells.
2. M form which is common in anaerobic cells.

(Kaplan and Everse, 1972). It was suggested that the catalytic activities of the two types are controlled by protein structural changes. Indeed pyruvate inhibition is highest with the H type (ie. aerobic type) and this inhibition is due to the formation of a temporary ternary complex between the oxidised coenzyme, pyruvate and the enzyme itself -

viz.

$$NADH + Pyruvate \xrightarrow[LDH]{} Lactate + NAD^+$$

competition

$$NAD^+ + Pyruvate + LDH \longrightarrow Ternary\ complex$$

This complex is much more readily formed in the 'aerobic' or H isomer. In behaviour, then the M isomer is equivalent to a pyruvate reductase and the H isomer a lactic dehydrogenase.

$$\text{ie. Pyruvate} \underset{\text{M isomer}}{\overset{\text{H isomer}}{\rightleftharpoons}} \text{Lactate}$$

Kaplan and Everse concluded that the existence of the ternary complex (H type ternary complex) depends on the NAD^+:NADH ratios. Therefore the H type is the regulatory enzyme since it is the type forming the ternary complex.

In a study of LDH McManmon (1969) found some induction on flooding in several non helophytes, but the activities were small and the differences not significant. He also found a small change in one helophyte. Similarly, Crawford and Tyler (1969) found that lactic acid was absent, or present to only a very minor degree, in plants as a result of flooding their root systems.

IV. GENERAL DISCUSSION - THE ENERGETICS OF ANAEROBIOSIS

An overall analysis of metabolic reactions to anoxia in seeds can be based upon an energetic analysis. This approach is justified because the biological role of glycolysis and of fermentation is to provide energy in the form of high energy phosphorus compounds (eg. ATP) and NAD^+ in order to maintain growth when gaseous oxygen is not available.

Normally oxygen is utilized at the terminal oxidase systems in the reduction of NADH with generation of ATP and NAD^+

$$NADH + H^+ + 3ADP + 3Phosphate \longrightarrow NAD^+ + H_2O + 3ATP$$

Thus ATP is available as a high energy compound and NAD^+ is available to oxidise further metabolic reactions.

Thus Table 60 summarise some of the previously recorded products which accumulate under anoxia, in plants, in relation to their energy yield. High ehergy phosphorus compounds (ATP) and oxidised hydrogen carriers are recorded (ie. NAD^+ and $NADP^+$). The most important observation is that all of the reaction chains either recycle NAD^+ or generate ATP. The successful nature of a metabolic shift depends upon both the generation of energy and the

oxidation of hydrogen carriers under anoxia. It might therefore be expected that both criteria would be satisfied in each case. Shikimic acid is then the exception to this rule, in that no ATP is generated. However, as Tyler and Crawford (1969) demonstrated, this product is mainly accumulated during the winter months in the rhizomes of Iris pseudacorus L. grown under flooded conditions. It is likely that growth is non existent and this may be a mechanism to stave off death rather than to maintain growth (ie. this allows $NADP^+$ recycling). Evidence in favour of this argument is given by the rapid metabolism of shikimate and the appearance of other products (eg. malate) of anoxia under flooding in the summer months. This malate accumulation is thought to generate energy in the form of ATP (Davies etal, 1974).

However, during anaerobiosis there may not only be a rate limiting process, but there may also be a quantity limiting process. For example in the case of glucose conversion to glycerol a 50% conversion rate to glycerol has been reported in African Trypanosomes (vonBrand, 1966). Crawford (1972) schematically links NADH reduction, on formation of glycerol with NAD^+ oxidation in the glycolytic pathway. In this reaction scheme then,

theoretically, for each molecule of glycerol formed one molecule of one of the intermediates of glycolysis must be formed. This emphasises the essential nature of reactions to anoxia. That is that a pool of end products is formed and the quantitative relationship between these products determines the success of the organism under anoxia. Thus if the products are easily remetabolised and are non toxic, then an organism with a metabolic shift in the direction of these products under anoxia is likely to survive ie. diversification of end products of anaerobic metabolism.

An examination of glycolysis, starting with glucose as the substrate through to phosphoenol-pyruvate, shows no net gain of ATP, while NAD^+ is reduced to NADH. Therefore, glycolysis to this point is self sufficient assuming a supply of substrate and of oxidised NAD^+.

ie. Glucose + 2ATP + 2NAD $\rightarrow$ Phosphoenolpyruvate + 2ATP + 2NADH + $2H^+$

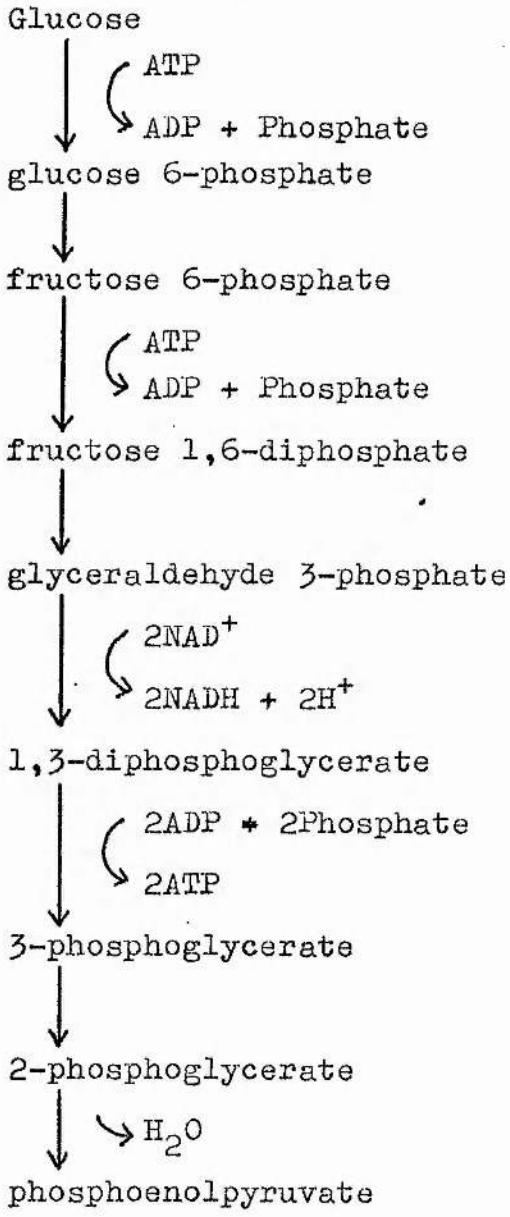
However, as the glycolytic pathway proceeds (also known as the Embden-Meyerhoff pathway) so the available NAD^+ will be depleted, which would be severely rate limiting if not regenerated. It is at this point that the energetics of anaerobic respiration become critical because phosphoenol-

Table 60.

Examples of reports from the literature of NAD^+ recycling and high energy phosphorus compound generation (eg. ATP) under anaerobiosis in a number of plant tissues.

End Product	Recycling of NAD^+	Generation of high energy compound	Plant	Reference 1. end product 2. energy yield
lactic acid	yes	yes	bean (French)	1. Sherwin and Simon (1969) 2. Bronk, 1973
glycerol	yes	yes	alder	1. Crawford, 1972 2. Crawford, 1972
alcohol	yes	yes	tomato	1. Fulton and Erikson, 1964 2. Bronk, 1973
shikimic acid	yes	no	Iris pseudacorus L	1. Crawford and Tyler, 1969 2. Crawford and Tyler, 1969
malic acid	yes	yes	bog plants	1. Crawford and Tyler, 1969 2. Davis etal, 1974

Figure 49. The pathway of glycolysis taking glucose as the substrate through to phosphoenol-pyruvate (as taken from standard texts in biochemistry).



pyruvate has two frequently reported fates under anoxia. Normally it is channeled into pyruvate which is then itself reduced to regenerate NAD^+, but there is evidence for a shift to oxaloacetate and hence malate in many species (Crawford and Tyler, 1969). Further evidence suggests that the malic acid may be converted to pyruvate subsequently (eg. in flood intolerant species - McManmon and Crawford, 1971).

The energetics of these two reactions may be summarised

1. Phosphoenolpyruvate + ADP + phosphate $\xrightarrow{\text{pyruvate kinase}}$ Pyruvate + ATP

Or

2a. Phosphoenolpyruvate $\underset{\text{PEPcarboxylase}}{\rightleftharpoons}$ oxaloacetate

2b. Oxaloacetate + NADH $\underset{\text{MDH}}{\rightleftharpoons}$ Malate + NAD^+ + H^+

2c. Malate + NADPH $\underset{\text{malic enzyme}}{\rightleftharpoons}$ Pyruvate + NADP + CO_2

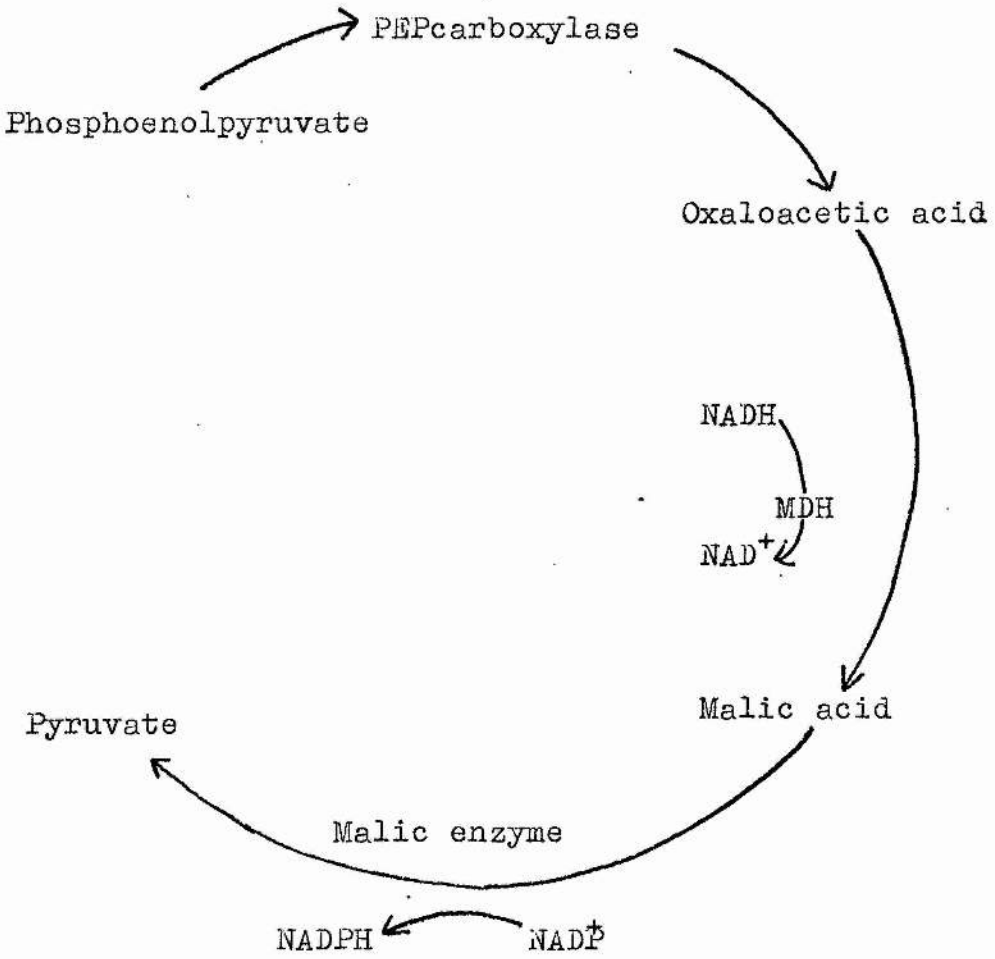
Alternatively for 2a.

Phosphoenolpyruvate + ADP + CO_2 $\underset{\text{PEPcarboxykinase}}{\rightleftharpoons}$ Oxaloacetate + ATP

Thus there is a net gain of ATP on the production of pyruvate by pyruvate kinase, while there is also a gain via the oxaloacetate - malate shift

Figure 50.

Reaction steps for shift via malic acid to pyruvate.



where phosphoenolpyruvate carboxykinase is the active enzyme. However, McManmon and Crawford (1971) in a metabolic scheme for flooding tolerance related oxaloacetate formation to the enzyme phosphoenol-pyruvate carboxylase (as found by Ting and Dugger, 1965), but although this enzyme was found to be active in the species tested, there was no correlation between its distribution and flooding behaviour in the species examined. A possible reason for this confusion is the existence of a synergistic effect between PEP carboxylase and PEP carboxykinase, where the former enzyme increases the activity of the latter enzyme (Vennesland, 1963). McManmon and Crawford (1971) found PEP carboxykinase activity in only one species and concluded that its general significance in flooding tolerance was doubtful. Davis etal (1974) believe the PEP carboxylase reaction to be unlikely on energetic grounds and suggest that PEP carboxykinase may be involved. However, in flood tolerant plants where malic acid is often the end product of anaerobic respiration, NAD^+ is regenerated by the oxaloacetic acid- malic acid reaction.

Because of the high growth rates of these species, it seems likely that ATP must be produced in the PEP to oxaloacetate step as there would

otherwise be no net gain of ATP during anaerobic growth.

$$PEP + CO_2 + ADP \underset{pck}{\rightleftharpoons} \text{Oxaloacetate} + ATP$$

(pck = PEPcarboxykinase)

Even during fermentation there is a slow net gain of ATP from the PEP to pyruvate steps with regeneration of NAD^+ on reduction of pyruvate to alcohol.

In flood intolerant plants, if there is a shift via oxaloacetate and malate leading to pyruvate, there will be no gain of oxidised hydrogen carrier (NADH is oxidised in the conversion to malate but $NADP^+$ is reduced on conversion of malate to pyruvate)(Figure 50) but there would be a similar gain in ATP energy as in fermentation processes.

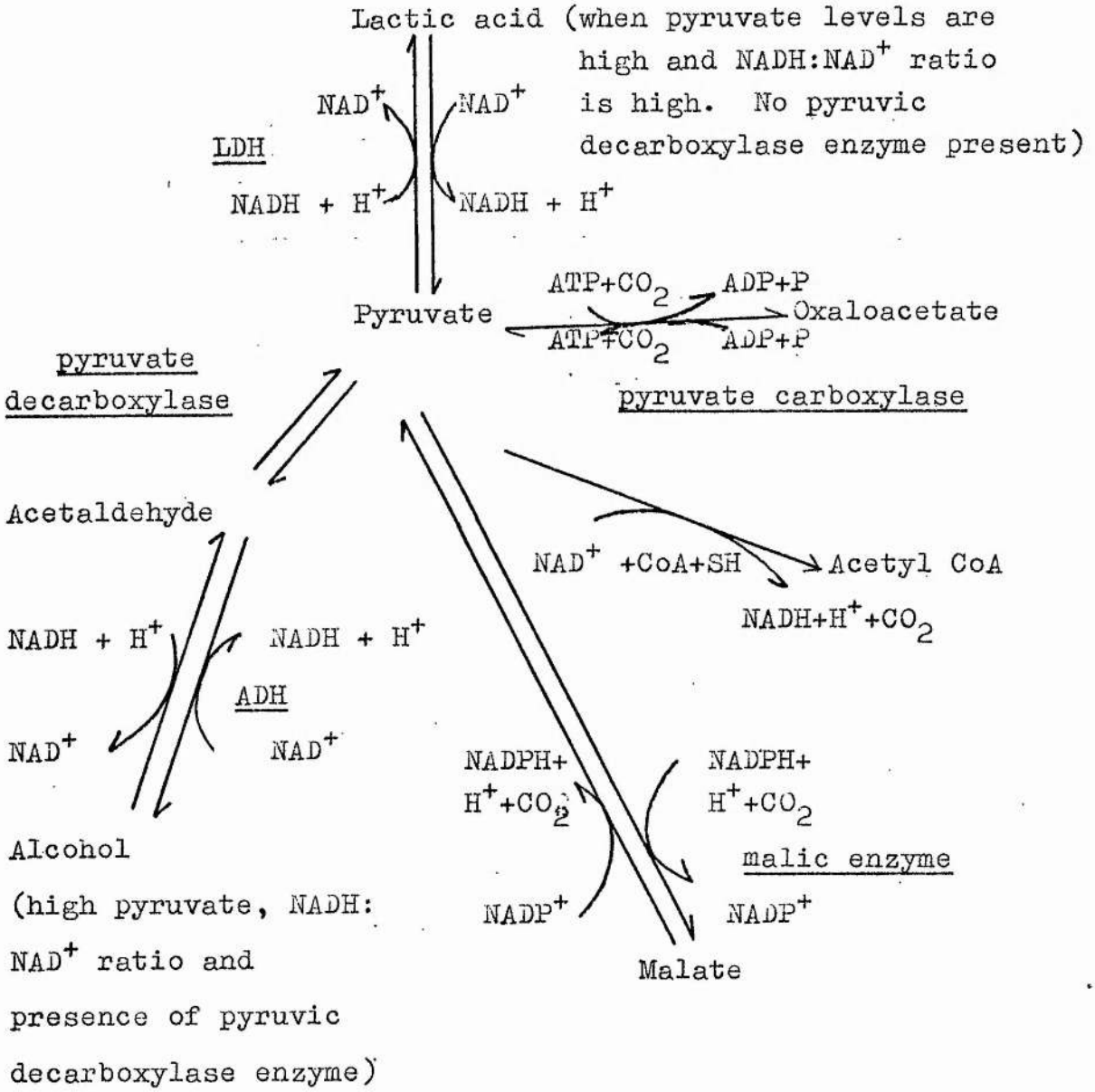
Therefore, NAD^+ has to be regenerated by the reduction of pyruvate in one of the ways shown in Figure 51. Of these alternative pathways, the pathway from pyruvate to oxaloacetate is unlikely under anoxia, because it destructively utilizes ATP. Also the pathway to Acetyl Co A and thence the TCA cycle (Krebs cycle) is limited because it is an NAD^+ requiring process.

This leaves three real possibilities-

1. malic acid as an end product.
2. alcohol.
3. lactic acid.

Figure 51.

Outline of alternative fates of pyruvate under anoxia.



Malic acid

As a direct metabolite of pyruvate malic acid is unlikely. This is because of the very large differences found by McManmon and Crawford (1971) in the activity of malic enzyme between flood tolerant and flood intolerant species. Although they reprted the absence of malic enzyme in flood tolerant plants, Davis etal found the enzyme to be active. The difference is probably quantitative and this suggests that if the reaction did run pyruvate to malate then flood intolerant plants would accumulate malic acid in preference to tolerant species. This has not been found to be the case (eg. Crawford and Tyler, 1969).

Both alcohol and lactic acid accumulation are likely on energetic grounds with their regeneration of NAD^+ (also ATP generation to pyruvate)

viz.

1a. Pyruvic acid $\rightleftharpoons$ Acetaldehyde + CO_2
(pyruvic decarboxylase)

1b. Acetaldehyde + NADH $\rightleftharpoons$ Alcohol + NAD^+
(alcohol dehydrogenase)

2. Pyruvic acid + NADH $\rightleftharpoons$ Lactic acid + NAD^+
(lactic dehydrogenase)

The regulation of enzyme activity under anaerobiosis

In the foregoing discussion, anaerobic pathways have been considered with reference to metabolism of reserves within the seed. However, the existence and relationship of metabolites and energy rich compounds will be determined by the presence and activity of the enzymes involved. Thus in a study of selected enzymes differing activities were shown between soaking tolerant and soaking sensitive species. Generally ADH and invertase activities were higher and MDH activities lower in species sensitive to soaking injury, after experimental soaking treatments.

It is this plasticity of enzymes which may hold the key to enzymic control of soaking tolerance. Substrates, intermediates and products may all have important controlling effects on enzyme activity. This is illustrated by reference to some of the theoretical points at which control mechanisms may operate. For example pyruvate to lactic acid will be controlled by NAD^+ to NADH ratios. This is supported by the change in ratio (increase) on removal of pea seedlings from anaerobic to aerobic conditions (Effer and Ranson, 1967). Phosphoenol-pyruvate conversion to pyruvate will similarly be controlled by ADP to ATP ratios. Thus glycolysis

in germinating pea seeds is inhibited by ATP (Mayer and Mapson, 1962; Mossberg, Mayer and Mapson, 1964), so that a high ratio of ADP to ATP will result during anaerobic germination.

Further control is often imparted by changes of the enzyme itself. A large number of enzymes exist in multiple forms, some of which may be rendered inactive by the action of inhibitors. Thus control may take the form of an allosteric control system (Monod, 1966) where an enzyme has a binding site for a regulatory molecule and a further catalytic site. However, a change in three dimensional shape may render the enzyme inactive, as in the case of combination with the regulatory molecule. The control will then be determined by the presence of substrate which will combine with the enzyme in preference to the allosteric inhibitor ie. substrate availability will stabilise the enzyme in the active form.

Individual steps may then be linked to reactions which supply the substrate and remove the products and therefore the accumulation of the final product in the reaction scheme will determine the overall rate of the metabolic sequence. For example in soaking sensitive species the final reaction (acetaldehyde to alcohol) of fermentation is favoured by a fall in

Km for ADH (with respect to acetaldehyde) causing an acceleration of the whole chain of reactions which similarly shift to compensate. In a bid to provide energy this reaction accelerates with a reversal of the Pasteur effect, sometimes referred to as the Crabtree effect (Bronk, 1973).

D. TEMPERATURE AND SOAKING INJURY

I. Introduction

Soaking injury varies with the temperature of soaking, and the latter part of this thesis examines two questions which arise. Firstly, whether submergence at low temperature has a more severe effect on injury, as is the case with low temperature imbibition on germination (Pollock and Toole, 1966). Secondly, whether an increase in temperature of soaking results in greater injury, because of an increase in the rate of anaerobic respiration, because it is a metabolic process and should therefore rise with temperature as a result of the Q_{10} effect.

II. LOW TEMPERATURE SOAKING INJURY

When the effect of low temperature ($0^{\circ}C$) (the treatments were maintained marginally above this temperature to prevent freezing) on underwater germination was examined, none of the species (including soaking tolerant species) germinated. At $10^{\circ}C$ good germination was recorded in all of the soaking tolerant species (probabilities by chance of the observed differences from $p = 2.7 \times 10^{-8}$ to $p = 4.6 \times 10^{-14}$) (Table 38).

Again the effect of a presoaking treatment was examined in each species. In the soaking tolerant species there was little evidence for enhanced injury (in subsequent germination counts) after low temperature soaking treatments. In the species susceptible to soaking injury, there was a lower germination count after low temperature imbibition (block diagrams in Figures 17 to 26). However, the effect on maize was very small; this species is the most tolerant of injury of the previously recorded susceptible group.

The apparent discrepancy between the underwater germination trials and germination counts taken after a presoaking treatment is probably due to the prolonged time required for germination at $0^{o}C$ in the underwater experiment. Indeed, none of the species germinated at this temperature, even if left in incubators for several weeks. Since the seeds used in the presoaking trials were all sown at the same time, this variable was eliminated in this experiment.

Damage as a result of low temperature ($0^{o}C$) was therefore enhanced only in species normally susceptible to soaking injury. Morinaga (1926) demonstrated underwater germination in several herbaceous species but found that this would only

occur if the temperature was adequate, although this may simply be a temperature requirement for a sufficient rate of metabolism for germination to commence. However, Pollock and Toole (1966) found that low temperature damage, as a result of low temperature imbibition, impaired germination. Polya (1961) using Populus alba seeds imbibed at 5°C from 0 to 60 minutes and then air dried found a distinct fall in germination (abnormalities were recorded where germination did occur eg. the presence of hypocotyl hairs, less firm rooting and inverse geotropism. These abnormalities are likely to reduce the survival chances of seedlings which do germinate).

This fall in germination with duration of low temperature treatment was more than additive with length of treatment. Figure 52 is based on data derived from Polya's paper and shows this relationship. Although this may not be directly comparable with the injury recorded in this thesis (Polya air dried his seeds after imbibition) it is striking that such a high order of damage occurred so rapidly. This is consistent with the theory proposed by Woodstock and Pollock (1965). They suggested that the mechanism of chilling injury was the result of physical injury to the seeds, in that at the very low rates

of respiration at low temperatures, membrane expansion is unable to compensate for the rapid rate of water uptake.

There is evidence for such a reduction in respiration rate at low temperature in a number of species eg. in lima beans (Woodstock and Pollock, 1965), in birds foot trefoil (Qualls and Cooper, 1968), in cotton (Hayman, 1969) and in beans (Opik and Simon, 1963; Orphanos and Heydecker, 1968).

This implies that the length of exposure to low temperature during imbibition (assuming the availability of water) will determine the extent of injury, as found by Polya, resulting from purely physical forces.

To ascertain whether low temperature soaking damage is related to the metabolic damage already discussed in the earlier part of this thesis (ie. at higher temperatures) experiments were conducted on four pea cultivars of differing soaking tolerance. A study of the enzyme invertase was used to monitor changes in sucrose and to gain further insight into the metabolism of the seed. No significant change was recorded in this enzyme at low temperature (Tables 44 and 45).

Alcohol accumulation was lower in low temperature

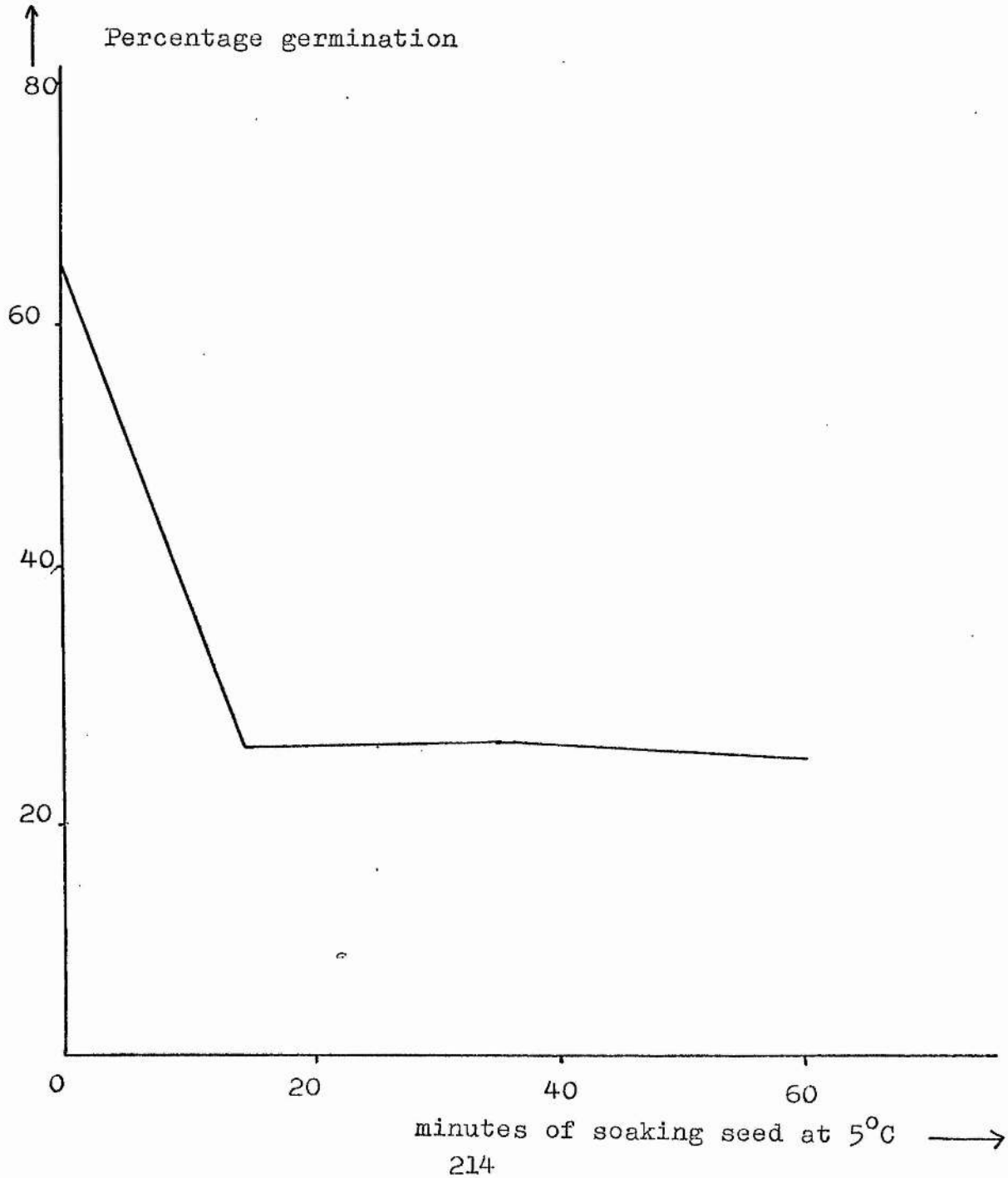
soaking treatments, while malic acid was relatively little affected by low temperature.

From the current experiments there appears to be no evidence in favour of enhancement of metabolic damage as a result of low temperature soaking treatments along the lines discussed in the earlier part of this thesis. The theory that low temperature (and hence low temperature soaking) damage can result from physical injury is strengthened by the work of Hayman (1969). He observed greater exudation rates from cotton seeds at low temperature, which presumably the result of membrane damage with consequent leakage from the seed. However, Christiansen (1968) claimed that death of seeds after chilling injury was due to a metabolic block; or a combination of physical injury with a metabolic block (Pollock, 1969; Obendork and Hobbs, 1970). It is true, of course, that physical injury could be the primary cause of damage resulting in a metabolic block directly as a result of tissue disintegration and leakage from the seed.

It is important to stress that in many reports of low temperature damage, secondary infection by pathogens was important. Thus Dickson (1923) found that attack of maize seeds by Gibberella zeae varied according to the temperature and water

Figure 52.

Percentage germination after soaking seeds of Populus alba for varying duration at 5°C. (Data taken from Polya 1961).



at germination. The fungus became more pathogenic at low temperatures (too low for successful germination of maize). Hayman (1969) similarly demonstrated pathogenic attack at low temperature, but attack by the pathogen was favoured as a result of increased sugar exudation by the seed. Exudation rates are often greater at low temperatures eg. in cotton (Hayman, 1969) andin peas (Perry and Harrison, 1970).

III. HIGH TEMPERATURE SOAKING INJURY

Underwater germination trials conducted at 10, 20 and 30°C showed a greater fall in germination at 30°C in the species least tolerant of soaking injury (Table 37). Similarly in experiments on germination after presoaking, high temperature made little difference to germination counts in the tolerant species but there was a marked interaction of temperature and duration of soaking in species previously recorded as susceptible to injury. High temperature in combination with a long presoaking treatment had the most severe effect on subsequent germination in these species (Tables 39 and 40).

DeBell and Naylor (1972) demonstrated a similar high temperature reduction in the germination of swamp tupelo seeds (Nyssa sylvatica). They attempted to germinate seeds in both soil submerged in flood stagnant water and in similar conditions with aeration of the floodwater at 15, 21 and 33°C. As the seeds failed to germinate, they tried a subsequent germination test under conventional conditions (the seed were underwater for several weeks in the previous experiment prior to their use in this experiment). A severe reduction in germination was recorded after 33°C soaking when compared with 21°C. Germina tion after soaking

in the aerated treatment was also reduced with temperature, but less so than in the flood stagnant treatment.

Mayer and Poljakoff-Mayber (1963) and Spragg and Yemm (1959) observed that temperature effects had less effect on respiration than expected because of the effect of the seed testa which inhibited gaseous exchange.

Respiration under nitrogen was compared in four pea cultivars at 10 and at 30°C to determine the Q_{10} for the reaction (Table 41). In the least tolerant cultivars QCO_2^n 10°C hour^{-1} was higher than in the more tolerant cultivars. Similarly at 30°C the least tolerant cultivars gave higher respiration rates under nitrogen. This compares with the earlier results where QCO_2^n was related to soaking tolerance at 25°C. In this case QCO_2^n rose at 30°C compared with 10°C as expected because respiration is a metabolic reaction and is therefore influenced by temperature.

Calculated Q_{10} (based on root of 10 to 30°C temperature range as described earlier) gave unexpected results. The more tolerant cultivars gave much higher Q_{10} values. However, the fact that the absolute respiration rates under nitrogen were lower in these varieties is of importance. The high Q_{10} implies that the differential

soaking tolerance of pea cultivars is lost as temperature of soaking rises above a threshold level.

Changes in storage material and its enzymic degradation were monitored by a study of the enzyme invertase in relation to temperature of soaking treatment. Invertase activity (both specific activity and activity per g seed) rose with temperature and both were negatively correlated at 30°C with germination after a 30°C soaking treatment for 24 hours (Tables 61 and 62 and Figures 53 and 54).

It has already been shown that a fall in the sucrose content of seeds as a result of soaking injury is indicative of the amount of damage to the seed. In sweet potato, on wounding, an increase in respiration reflected a decrease in sucrose content (Uritani and Kato, 1973), while Sacher etal (1963) related the synthesis of invertase in sugar cane stems to a glucose control system. Also in Avena segments sucrose stimulated growth and invertase activity (Kaufman etal, 1968) while Hawker (1971) concluded that invertase synthesis was probably induced by sucrose. He also found invertase to be the only enzyme catalysing sucrose breakdown. However, this effect was complicated by the existence of two isoenzymes, only one of which was inducible by sucrose.

Table 61.

The relationship between germination after 24 hours presoaking at 30°C and invertase specific activity after soaking seed at 30°C for 24 hours.in Pisum cvs.

Cultivar	Germination % of control	Invertase specific activity I.U. mg protein^{-1}
Gladstone	63.64	19.1
Meteor	50.00	31.3
Pioneer	42.11	45.0
Onward	35.19	45.8

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	458.971	1	458.971	38.55
Residual	23.809	2	11.905	
Total	482.780	3		p=.05

Figure 53.

Fitted regression line for invertase (I.U. g dry weight seed^{-1}) after presoaking seeds for 24 hours at 30°C against percentage germination after soaking seeds for 24 hours at 30°C (percentage of control or unsoaked seeds which germinated) in four pea cultivars of varing soaking tolerance.

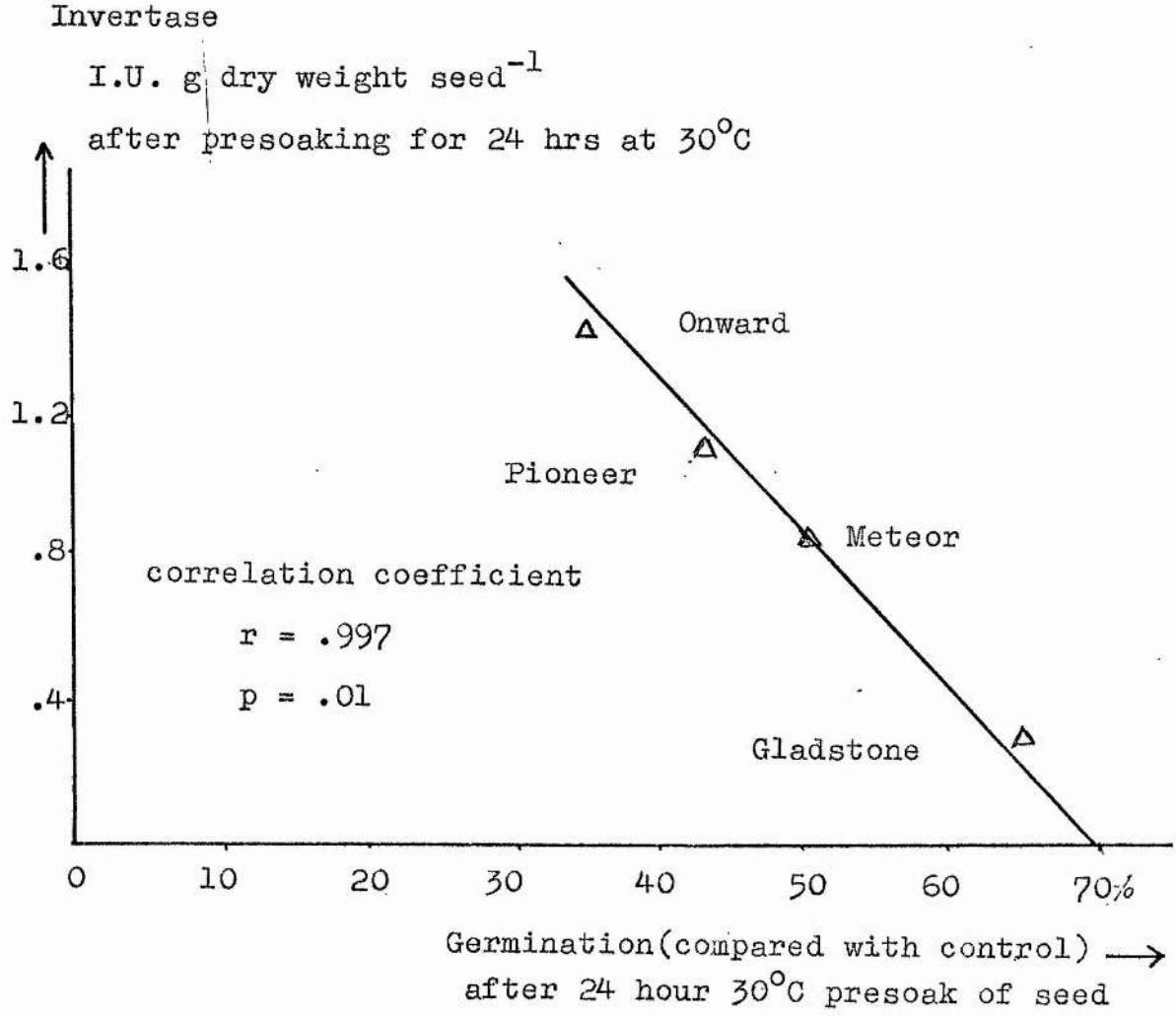


Figure 54.

Fitted regression line for inveratse (I.U. mg protein^{-1}) after presoaking seeds for 24 hours at 30°C against percentage germination after soaking seeds for 24 hours at 30°C (percentage of control or unsoaked seeds which germinated) in four pea cultivars of varying soaking tolerance.

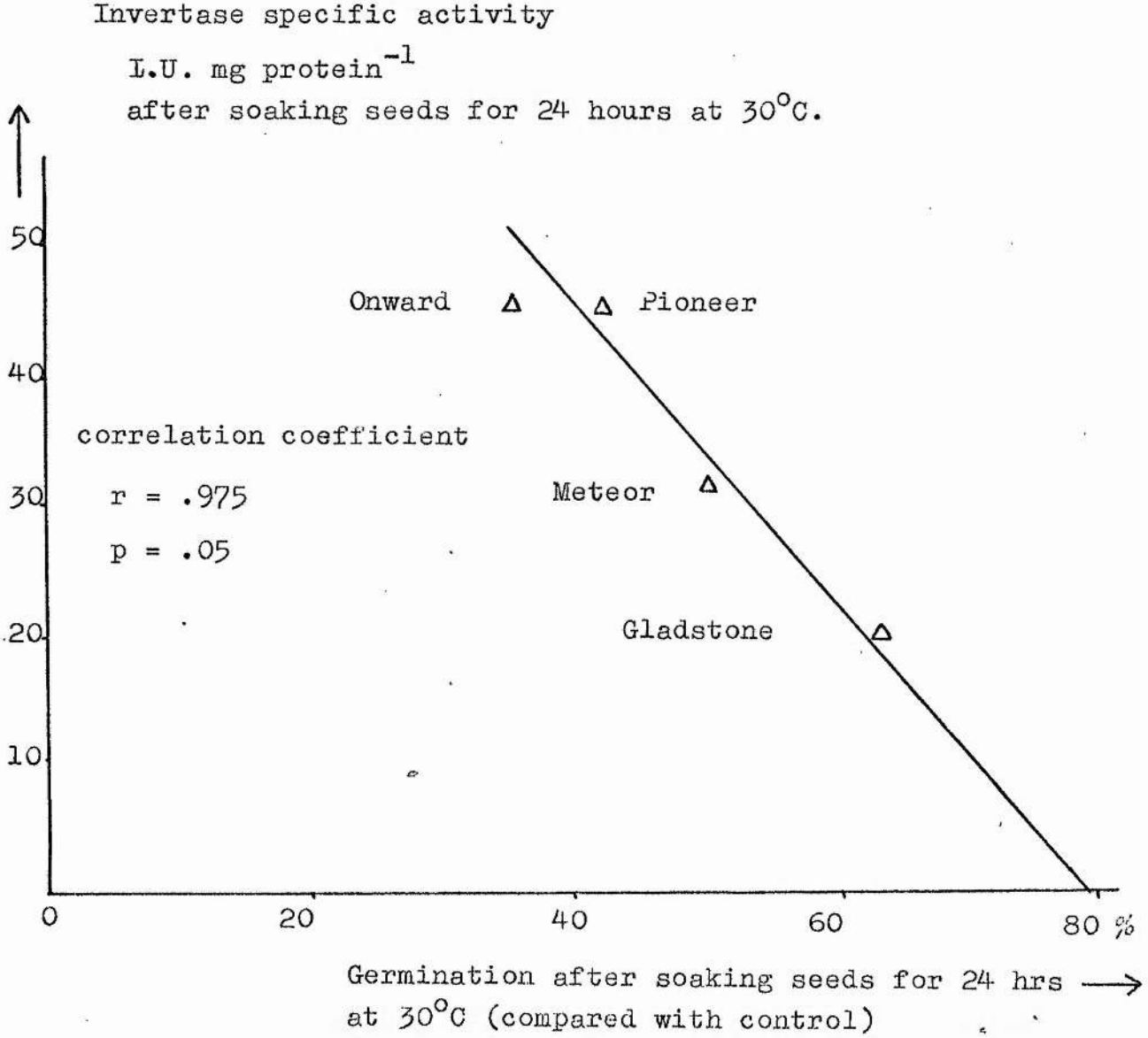


Table 62.

The relationship between germination after 24 hours presoaking at 30°C and invertase activity per g seed (I.U. g dry weight seed^{-1}).

Cultivar	Germination % of control	Invertase I.U. g dry weight seed^{-1}
Gladstone	63.64	.256
Meteor	50.00	.840
Pioneer	42.11	1.11
Onward	35.19	1.42

Regression analysis

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	.7283	1	.7283	336.2
Residual	.004333	2	.002166	
Total	.7326	3		p=.01

It may therefore be concluded that the loss of sucrose in susceptible species results from a change in the activity of the enzyme invertase, which increases in activity sharply with a rise in temperature, especially in the soaking sensitive pea cultivars.

A similar relationship exists between alcohol content of seeds and invertase activity. This also reflects soaking tolerance ie. higher alcohol contents were recorded in the more susceptible cultivars (Table 46, Figures 38 and 39). It may be concluded that higher alcohol content as a result of increasing temperature leads to greater seed meristem damage and that the metabolic shift to malate affords a comparative advantage in soaking tolerance of seeds. This shift holds good and is relatively unaffected by temperature. It is notable, however, that a significant fall in malic acid with temperature was recorded in one of the least tolerant cultivars (Pioneer, $p=.025$)(Table 48). In this case alcohol content became much accelerated at the expense of malic acid accumulation.

Calculated ethanol to malic acid ratios have been used as a criterion of soaking tolerance and again give a good indication for soaking injury in pea seeds. Germination shows a negative relationship

Table 63.

Metabolic and germination behaviours of four cultivars of Pisum sativum L. arranged by rank with a test for correlation. (Of the four values for each metabolic parameter, the lowest is ranked as one grading up to four in the cultivar with the highest value. Germination and malic acid content of seeds are ranked in the reverse order because they are negatively related to the other parameters - see earlier discussion).

Cultivar	Germination.	QCO_2^n	Sp.Act Invertase	Act seed Invert.	Alcohol.	Malate	Total
Gladstone	1	2	1	1	1	1	7
Meteor	2	1	2	2	2	2	11
Pioneer	3	3	3	3	4	3	19
Onward	4	4	4	4	3	4	23

Table 64.

The relationship between the observed correlation between germination and metabolic classes after presoaking seeds of four cultivars of Pisum sativum L. for 24 hours at 30°C.

Cultivar	Observed row total	Theoretical maximum
Gladstone	7	6
Meteor	11	12
Pioneer	19	20
Onward	23	24

against ethanol to malic acid ratios.

The effects of high temperature soaking injury on the four pea cultivars have been collated in a summary table (Table 63). Although not based on a standard statistical test the good correlation between germination after presoaking seeds at 30°C and the various metabolic classes in the four pea cultivars is striking. If a perfect relationship is assumed, then the expected results would be as shown in Table 64.

It is therefore reasonable to conclude that the relationship is very strong indeed between soaking injury, anaerobic respiration rate, invertase activity, alcohol and malic acid accumulation (germination and malic acid accumulation being negatively correlated with the other parameters) after 30°C soaking treatments. This may be taken as confirmatory evidence that at high temperatures, but not at low temperatures, ..., there is an increase in the rate of glycolysis and that accumulation of anaerobic end products accounts for interspecific and intraspecific differences.

SUMMARY

Seeds of a range of crop species were classified according to their ability to germinate after presowing soaking treatments of varying severity.

1. Those species most susceptible to injury exhibited the highest respiration rates under nitrogen after soaking their seeds ($p=.01$ for fall in germination after 72 hours seed soaking against QCO_2^n).

2. Where anaerobic respiration was highest, the utilization of seed reserves was greater as shown by the fall in sucrose content of the seed on soaking for 96 hours ($p=.001$ for rate of respiration under nitrogen against loss of sucrose from the seed per hour).

3a. The increase in alcohol content of seeds after soaking seeds for 96 hours (compared with controls) was positively correlated with respiration under nitrogen on soaking ($p=.05$).

3b. This increase in alcohol content of seeds was also positively correlated with the fall in sucrose content of seeds on soaking (fall in sucrose per hour)($p=.05$).

4a. Malic acid content of seeds after soaking for 96 hours (compared with controls) was negatively

correlated with respiration under nitrogen on soaking seeds (p=.001).

4b. Malic acid content of seeds after soaking (compared with controls) was also negatively correlated with the fall in sucrose content (per hour) of seeds on soaking (p=.001).

4c. Malic acid content of seeds on soaking for 96 hours was negatively correlated with alcohol content of seeds after soaking for 96 hours when expressed as increases compared with controls. (p=.02).

5. The percentage of lactic acid, when expressed as the percentage of alcohol + malic acid + lactic acid after soaking seeds for 96 hours, was negatively correlated with respiration under nitrogen on soaking seeds (p=.007).

6a. The activity of alcohol dehydrogenase was highest in species least tolerant of soaking injury.

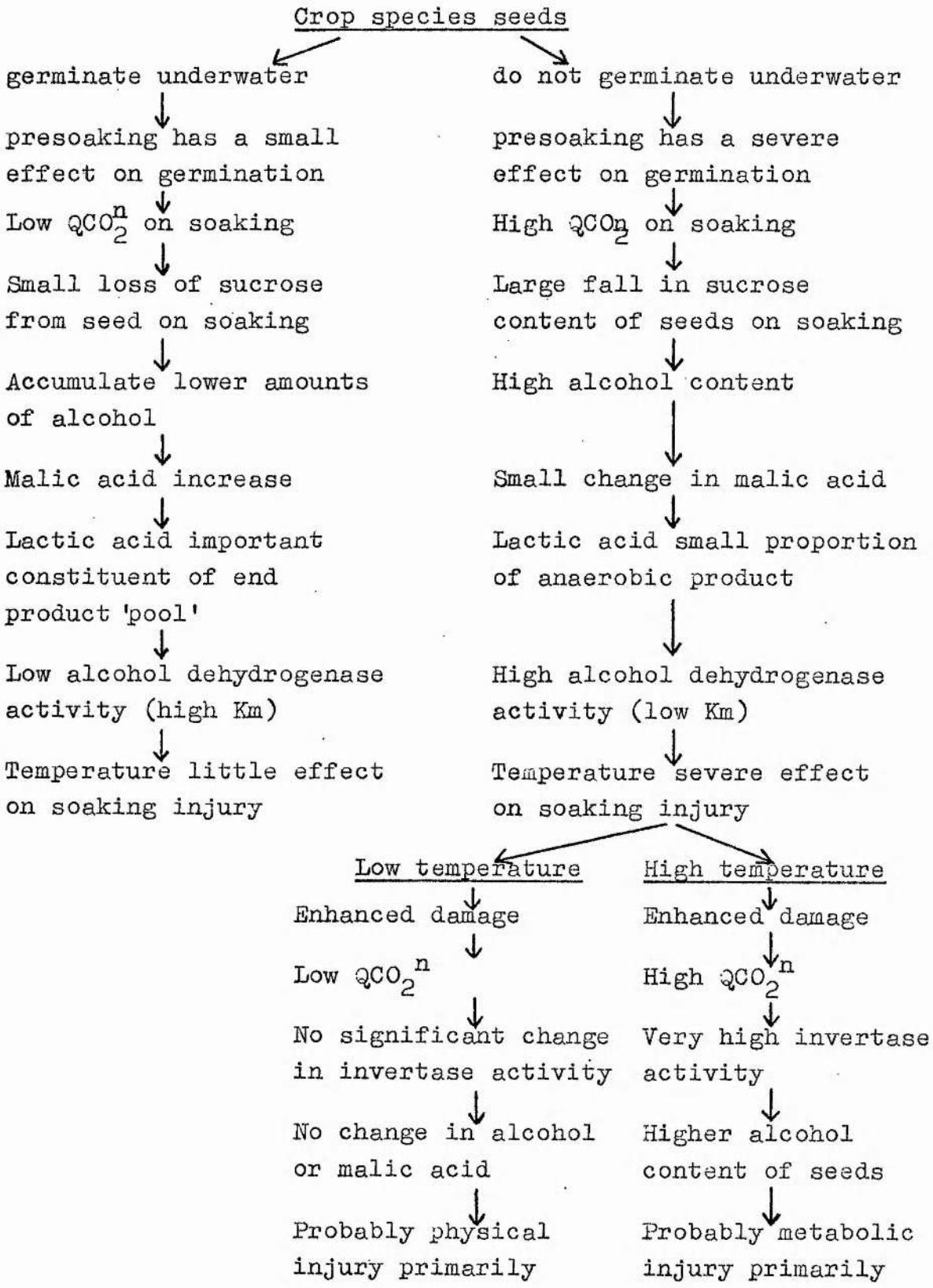
6b. Michaelis constants for ADH with respect to acetaldehyde were low in species intolerant of soaking but higher in species tolerant of this treatment.

7a. Enhanced damage as a result of low and high temperature soaking treatments were recorded only in species which were normally susceptible to soaking injury.

7b. Metabolic injury in Pisum sativum L. followed a similar course at high temperature to the above studies but not at low temperatures.

7c. Studies on the activity of the enzyme invertase demonstrated grossly enhanced activity with a rise in temperature. This enhanced activity was also related to germination counts.

Figure 55. Conclusion to thesis in diagrammatic form



APPENDIX

I. Abbreviations used in text

ml	millilitre
l	litre
mg	milligram
g	gram
^{o}C	degrees centigrade
min	minute
cm	centimeter
M	molar
hr	hour
nm	nanometer
μ	micro
$^{-1}$	per
%	percent
rpm	revolutions per minute
dry wt	dry weight
fr/gl	fructose + glucose
GasPak	anaerobe jar system manufactured by Bioquest
Tris-HCl	buffer made up to pH 8.0, 0.1M tris.
Q_{10}	change in reaction rate for 10° rise in temperature
RQ	respiratory quotient
QO_2	oxygen uptake 1 g dry weight^{-1}hr^{-1}

QCO_2	carbon dioxide evolution 1 g dry weight^{-1}hour^{-1}
QCO_2^n	respiration rate under nitrogen, 1 g dry weight^{-1}hour^{-1}
ADH	alcohol dehydrogenase
MDH	malic dehydrogenase
LDH	lactic dehydrogenase
Km	Michaelis constant
I.U.	International unit
cv	cultivar
p	probability of observed event by chance occurrence
r	correlation coefficient
S.D.	standard deviation
t	calculated value for students t test
F	calculated value for F test
N.S.	not significant

APPENDIX II. STATISTICAL APPENDIX

II. Method used for the determination of binomial probabilities for germination of seeds underwater and germination on moist filter paper.

If the probability of any seed germinating on moist filter paper is assumed to be Pa and the probability of any seed germinating underwater is assumed to be Pw, then is the binomial Pa=Pw true? ie. is there an equal probability that seeds will germinate underwater or on moist filter paper?

If Pa and Pw are amalgamated ie. based on the assumption that there is no effect due to soaking, then the probability of the observed results would be

$$\frac{\text{Factorial number of seeds}}{\text{Factorial number of seeds germinating on moist filter paper x factorial number of seeds failing to germinate on moist filter paper.}}$$

$$X \quad \frac{\text{Factorial number of seeds}}{\text{Factorial number of seeds germinating underwater x factorial number of seeds failing to germinate underwater.}}$$

$$X \quad \frac{\text{Factorial number germinated (water + control) x factorial number failing to germinate}}{\text{Factorial number which could germinate (maximum).}}$$

The above equation gives the probability of the exact split between underwater and control germination observed. For example in rice where 25 out of 30 seeds germinated underwater while 24 out of 30 germinated on moist filter paper. The probability of this ratio occurring by chance (assuming an equal probability for germination underwater and on moist filter paper) is then given by -

$$\frac{30!}{25!5!} \times \frac{30!}{24!6!} \times \frac{49!11!}{60!} = .25$$

However, we wish to know not only the probability of the exact split observed but the probability of the observed result or of one more extreme. In the above example the total germination was 49 seeds which germinated in the ratio 25:24 (water:control). The next more extreme case would be 26:23 (total = 49 seeds germinating) and the probability may be calculated accordingly.

Table 65 shows a worked example where the probabilities are summated for all the possible numbers of seeds germinating underwater (25 to 30) ie. this is the probability that, due to chance occurrence, 25 or more seeds will germinate underwater(out of a total of 49 seeds divided between the two treatments).

Table 65. Worked example using the method for determining binomial probability for germination of seeds underwater on on moist filter paper.

eg. For rice where 25 seeds out of 30 germinated underwater and 24 germinated on moist filter paper.

$$\frac{30!}{25!5!} \times \frac{30!}{24!6!} \times \frac{49!11!}{60!}$$

a) probability of 25 seeds germinating underwater =.25

$$\frac{30!}{26!4!} \times \frac{30!}{23!7!} \times \frac{49!11!}{60!}$$

b) probability of 26 seeds germinating underwater =.168

$$\frac{30!}{27!3!} \times \frac{30!}{22!8!} \times \frac{49!11!}{60!}$$

c) prdbability of 27 seeds germinating underwater =.0693

$$\frac{30!}{28!2!} \times \frac{30!}{21!9!} \times \frac{49!11!}{60!}$$

d) probability of 28 seeds germinating underwater =.0182

$$\frac{30!}{29!1!} \times \frac{30!}{20!10!} \times \frac{49!11!}{60!}$$

e) probability of 29 seeds germinating underwater =.0026

$$\frac{30!}{30!} \times \frac{30!}{19!11!} \times \frac{49!11!}{60!}$$

f) probability of 30 seeds germinating underwater =.00016

The probability of 25 or more seeds germinating under water by chance is then given by a+b+c+d+e+f (p=.50)

Additional statistical data referred to in text

Table 66. Regression analysis for oxygen uptake, carbon dioxide evolution, respiration under nitrogen and respiratory quotient in Pioneer pea seeds after soaking underwater from 0 to 48 hours.

	Source of variation	Sum of squares	Degrees freedom	Mean square	F
QO_2	Regression	.000094	1	.000094	.01661
	Residual	.01698	3	.00566	
	Total	.01708	4		N.S.
QCO_2	Regression	.008108	1	.008108	1.8014
	Residual	.01350	3	.004501	
	Total	.02161	4		N.S.
QCO_2^n	Regression	.02793	1	.02793	41.6230
	Residual	.002013	3	.000671	
	Total	.02294	4		p=.01
RQ	Regression	.09242	1	.09242	1.3019
	Residual	.2130	3	.07099	
	Total	.3054	4		N.S.

Table 67.

Regression analysis for oxygen uptake, carbon dioxide evolution, respiration under nitrogen and respiratory quotient in Meteor pea seeds after soaking underwater from 0 to 48 hours.

	Source of variation	Sum of squares	Degrees freedom	Mean square	F
QO_2	Regression	.002940	1	.002940	3.1410
	Residual	.002807	3	.000936	
	Total	.005747	4		N.S.
QCO_2	Regression	.01316	1	.01316	2.6650
	Residual	.01418	3	.004937	
	Total	.01934	4		N.S.
QCO_2^n	Regression	.01595	1	.01595	14.1034
	Residual	.003392	3	.001131	
	Total	.01934	4		p=.05
RQ	Regression	.04294	1	.04294	.2324
	Residual	.5543	3	.1848	
	Total	.5973	4		N.S.

Table 68.

Regression analysis for oxygen uptake, carbon dioxide evolution, respiration under nitrogen and respiratory quotient in rice seeds after soaking underwater for 0 to 48 hours.

	Source of variation	Sum of squares	Degrees freedom	Mean square	F
QO_2	Regression	.0001467	1	.0001467	82.4101
	Residual	.00000535	3	.00000178	
	Total	.0001520	4		p=.01
QCO_2	Regression	.006854	1	.006854	11.3644
	Residual	.001809	3	.0006031	
	Total	.008663	4		p=.05
QCO_2^n	Regression	.004493	1	.004493	8.6697
	Residual	.001555	3	.0005183	
	Total	.006048	4		N.S.
RQ	Regression	6.9068	1	6.9068	1.6323
	Residual	12.6942	3	4.2314	
	Total	19.6010	4		N.S.

Table 69.

Regression analyses for the effect of duration of presoaking treatment of seeds (in hours) against the accumulation of alcohol in rice and in carrot seeds. (0,24, 48 and 96 hour treatments have been transfomed to 0, 1, 2 and 4 respectively for ease of analysis).

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
rice	Regression	25.1586	1	25.1586	7.9381
	Residual	6.5905	2	3.2952	
	Total	32.7490	3		N.S.
rice control	Regression	.5764	1	.5764	.009710
	Residual	118.727	2	59.3636	
	Total	119.304	3		N.S.
carrot	Regression	2846.736	1	2846.736	.8931
	Residual	6375.2589	2	3187.6294	
	Total	9221.9953	3		N.S.
carrot control	Regression	137.1437	1	137.1437	3.4865
	Residual	78.6724	2	39.3362	
	Total	215.8161	3		N.S.

Table 70.

Regression analyses for the effect of duration of presoaking treatment of seeds (in hours) against accumulation of alcohol in lettuce and radish seeds. (using 0, 1, 2, 4 transformation as in Table 69).

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
lettuce	Regression	31.6044	1	31.6044	.4371
	Residual	144.6202	2	72.3101	
	Total	176.2246	3		N.S.
lettuce	Regression	745.7660	1	745.7660	3.7362
control	Residual	399.2152	2	199.6076	N.S.
	Total	1144.9813	3		
radish	Regression	64.3422	1	64.3422	2.8082
	Residual	45.8245	2	22.9122	
	Total	110.1667	3		N.S.
radish	Regression	.9667	1	.9667	.7933
control	Residual	2.4370	2	1.2185	N.S.
	Total	3.4037	3		

Table 71.

Regression analyses for the effect of duration of presoaking treatment of seeds against accumulation of alcohol in maize and broad bean seeds. (Using 0, 1, 2, 4 transformation for duration of soaking as in Table 69).

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
maize	Regression	869.6522	1	869.6522	17.1014
	Residual	101.7054	2	50.8527	
	Total	971.3576	3		N.S.
maize	Regression	3.5013	1	3.5013	.01429
control	Residual	489.9648	2	244.9824	
	Total	493.4661	3		N.S.
broad	Regression	391.2952	1	391.2952	1.4793
bean	Residual	529.0332	2	264.5166	
	Total	920.3283	3		N.S.
broad	Regression	398.3240	1	398.3240	7.0494
bean	Residual	113.0100	2	56.5050	
control	Total	511.3340	3		N.S.

Table 72.

Regression anlyses for the effect of duration of presoaking treatment of seeds against accumulation of alcohol in seeds of Meteor and Onward pea cultivars. (using transformation for duration of soaking treatment as in Table 69).

Cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Meteor	Regression	24.6632	1	24.6632	.002546
	Residual	19374.764	2	9687.3821	
	Total	19399.427	3		N.S.
Meteor control	Regression	378.9287	1	378.9287	.1890
	Residual	4009.5740	2	2004.7870	
	Total	4388.5027	3		N.S.
Onward	Regression	4604.6051	1	4604.6o51	3.0145
	Residual	3054.9242	2	1527.4621	
	Total	7659.5293	3		N.S.
Onward control	Regression	394.1562	1	394.1562	.2460
	Residual	3204.4781	2	1602.2391	
	Total	3598.6343	3		N.S.

Table 73.

Regression analyses for the effect of duration of presoaking treatment of seeds against accumulation of alcohol in seeds of pea cultivars Pioneer and Gladstone (Using transformations as in Table 69).

Cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Pioneer	Regression	1814.1552	1	1814.1552	.2152
	Residual	16858.5045	2	8429.2523	
	Total	18672.6597	3		N.S.
Pioneer control	Regression	273.4958	1	273.4958	.06569
	Residual	8326.7997	2	4163.3998	
	Total	8600.2955	3		N.S.
Gladstone	Regression	.09777	1	.09777	.000012
	Residual	15723.2056	2	7861.6028	
	Total	15723.3034	3		N.S.
Gladstone control	Regression	321.5185	1	321.5185	.1753
	Residual	3668.0485	2	1834.0243	
	Total	3989.5671	3		N.S.

Table 74.

Regression analysis for duration of soaking treatment of seeds against malic acid content in seeds of rice, carrot, lettuce and radish. (Using transformations as in Table 69).

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
rice	Regression	.08147	1	.08147	.1311
	Residual	1.2428	2	.6214	
	Total	1.3243	3		N.S.
carrot	Regression	37.6985=	1	39.6985	.2449
	Residual	307.8656	2	153.9328	
	Total	345.5641	3		N.S.
lettuce	Regression	6.7315	1	6.7315	.4832
	Residual	27.8620	2	13.9310	
	Total	34.5935	3		N.S.
radish	Regression	7.2387	1	7.2387	1.6965
	Residual	8.5338	2	4.2669	
	Total	15.7725	3		N.S.

Table 75.

Regression analysis for duration of soaking treatment of seeds, against malic acid content in seeds of maize, broad bean, Meteor pea and Onward pea. (Using transformations as in Table 69).

Species /cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
maize	Regression	.2076	1	.2076	6.9617
	Residual	.05964	2	.02982	
	Total	.26722	3		N.S.
broad bean	Regression	1.4333	1	1.4333	133.0414
	Residual	.02155	2	.01077	
	Total	1.4548	3		p=.01
Meteor pea	Regression	.6223	1	.6223	19.0331
	Residual	.06539	2	.03270	
	Total	.6877	3		p=.05
Onward pea	Regression	.1005	1	.1005	6.6641
	Residual	.03015	2	.01508	
	Total	.1306	3		N.S.

Table 76.

Regression analysis for duration of soaking treatment of seeds, against malic acid content in seeds of Pioneer and Gladstone peas (Using transformations as in Table 69).

Cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Pioneer	Regression	.8918	1	.8918	33.7189
	Residual	.05290	2	.02645	
	Total	.9447	3		p=.05
Gladstone	Regression	.8919	1	.8919	152.9571
	Residual	.01166	2	.005831	
	Total	.9036	3		p=.01

Table 77.

Regression analysis for duration for soaking treatment of seeds, against lactic acid content of seeds of rice, carrot, lettuce and radish (Using transformation as in Table 69).

Species	Source of variation	Sum of squares	Degrees freedom	Mean square	F
rice	Regression	45.3501	1	45.3501	4.9631
	Residual	18.2749	2	9.1374	
	Total	63.6250	3		N.S.
carrot	Regression	9061.9669	1	9061.9669	.6347
	Residual	28555.7529	2	14277.8764	
	Total	37617.7197	3		N.S.
lettuce	Regression	169.1163	1	169.1163	.8489
	Residual	398.4457	2	199.2229	
	Total	567.5620	3		N.S.
radish	Regression	47.9150	1	47.9150	6.6947
	Residual	14.3144	2	7.1572	
	Total	62.2294	3		N.S.

Table 78.

Regression analysis for duration of soaking treatment of seeds, against lactic acid content of seeds of maize, broad bean, Meteor pea and Onward pea (Using transformations as in Table 69).

Species/ cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
maize	Regression	2.6308	1	2.6308	2.5123
	Residual	2.0943	2	1.0471	
	Total	4.7250	3		N.S.
broad bean	Regression	.3255	1	.3255	.1467
	Residual	4.4377	2	2.2188	
	Total	4.7632	3		N.S.
Meteor pea	Regression	.3052	1	.3052	10.7743
	Residual	.05665	2	.02833	N.S.
	Total	.3619	3		
Onward pea	Regression	.009530	1	.009530	.02395
	Residual	.7957	2	.3979	
	Total	.8053	3		N.S.

Table 79.

Regression analysis for duration of soaking treatment of seeds, against lactic acid content of seeds of Pioneer and Gladstone peas. (Using transformations as in Table 69).

Cultivar	Source of variation	Sum of squares	Degrees freedom	Mean square	F
Pioneer	Regression	1.5596	1	1.5596	.9910
	Residual	3.1476	2	1.5738	
	Total	4.7072	3		N.S.
Gladstone	Regression	.06311	1	.06311	.08719
	Residual	1.4478	2	.7239	
	Total	1.5109	3		N.S.

Table 80.

regression analysis for increase in lactic acid content of seeds on soaking (ratio of lactic acid content after soaking seeds for 96 hours underwater to lactic acid content after germination on moist filter paper for 96 hours) against respiration under nitrogen (μl mg dry weight seed^{-1} hour^{-1}) in several crop species.

Source of variation	Sum of squares	Degrees freedom	Mean square	F
Regression	12.8145	1	12.8145	1.2841
Residual	59.8739	6	9.9790	
Total	72.6884	7		N.S.

APPENDIX III. ASSAYS REFERRED TO IN TEXT

1. Estimation of sugars

Somogyi's micro method for the estimation of reducing sugars.

This method depends on the reduction of alkaline cupric hydroxide to cuprous oxide. The cuprous oxide so formed is then oxidised by an excess of iodine in acid solution.

$$Cu_2O + I_2 + 2H_2SO_4 \longrightarrow 2CuSO_4 + H_2O + 2HI$$

Iodine is relatively unstable in solution, and iodine is therefore formed by the interaction of iodide with iodate in the reagent under acid conditions.

$$KIO_3 + 5KI + 3H_2SO_4 \longrightarrow 3I_2 + 3K_2SO_4 + H_2O$$

The amount of iodine used is estimated by titrating the excess with a standard solution of thiosulphate using starch as an indicator of the end point.

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

The following reagents are employed-

disodium hydrogen phosphate (anhydrous)	28g/l.
Rochelle salt	40g/l.
sodium sulphate (anhydrous)	180g/l.
copper sulphate	8g/l.
sodium hydroxide	100ml of a 1N solution.
potassium iodate	25ml of a 1N solution.

The Rochelle salt prevents the precipitation of copper in the alkaline solution. The anhydrous

sodium sulphate prevents the re-oxidation of cuprous copper, mainly by reducing the solubility of atmospheric oxygen in the solution. The above constitutes solution A.

2.5% potassium iodide - solution B.

2N sulphuric acid - solution C.

Method

Five mls of the sugar solution to be estimated and 5 mls of solution A were mixed in a boiling tube. The mixture was boiled for 15 minutes, cooled for 5 minutes and 2 mls of solution B added carefully down the side of the tube without mixing. 2 mls of solution C were then mixed in. 2-3 minutes were allowed for all of the cuprous oxide to dissolve.

The iodine liberated was then titrated against .005N thiosulphate (sodium) solution using starch as an indicator. The titration was repeated for a blank using distilled water in place of the sugar solution under assay.

The difference in the water blank and the sugar solution is used to calculate the amount of reducing sugar in the original 5 ml sample.

1 ml. of .005 N sodium thiosulphate in titre
=.135 mg of reducing sugar.

Non reducing sugars were estimated after inversion of the original solution by the enzyme invertase, in a warm water bath for 2 hours. Back titrations were then calculated as above.

2. Enzymic determination of ethanol

Reagents

Semicarbazide - glycine buffer. - 20g of $Na_4P_2O_7.10H_2O$, 5 g recrystallised semicarbazide hydrochloride and 1 g of glycine were dissolved in distilled water. 20 ml of 2N NaOH was added and the mixture diluted to 600 ml to a final pH of 8.8. Alcohol dehydrogenase (commercial preparation) and nicotinamide-adenine dinucleotide (commercial preparation) were also employed.

Method

$$\text{ethanol} + NAD^+ \underset{\text{ADH}}{\rightleftharpoons} \text{acetaldehyde} + NADH$$

The amount of NAD reduced to NADH is determined from the change in extinction (ΔE_{1cm}) at the end of the reaction, measured on a spectrophotometer at 334 nm. The ΔE_{1cm} for 1 mole of ethanol is 2.0. The test reactions were carried out as follows-

Cuvette	I	II	III	IV
buffer	1.0	1.0	1.0	1.0
extract	to make a total volume of 3 mls			
water				
NAD	.02	.02	.02	.02
ADH	.01	.01	.01	.01
total volume	3.0	3.0	3.0	3.0

The amount of extract and water was varied in order to give spectrophotometric readings on the most sensitive region of the spectrophotometer scale. The cuvettes were arranged so that cuvette II had twice as much extract as cuvette I, in order to check doubling of readings. Cuvette IV was always a control into which all of the reagents were placed with no extract added, while cuvette III contained extract and all the reagents with the exception of ADH.

3. Enzymic determination of malic acid

Reagents

Hydrazine - glycine buffer (0.4 M hydrazine; 1 M glycine, pH 9.5). 7.5 g of glycine, 5.2 g hydrazine sulphate and 0.2 g EDTA - $Na_2H_2.2H_2O$ were dissolved in distilled water and diluted to 100 mls. This stock solution is atable and small proportions were adjusted to pH 9.5 using 2N NaOH as required. Malic dehydrogenase (commercial preparation) and NAD (commercial preparation) were also employed.

Method

$$\text{malic acid} + NAD^+ \underset{\text{MDH}}{\rightleftharpoons} \text{oxaloacetate} + NADH$$

The assay was conducted in exactly the same way as

for ethanol, but using MDH instead of ADH and hydrazine-glycine buffer instead of semicarbazide-glycine buffer.

4. Enzymic determination of lactic acid

Reagents - Hydrazine-glycine buffer (the same buffer as for malic acid). Lactic dehydrogenase (commercial preparation) and NAD (commercial preparation).

Method

$$\text{lactacte} + NAD^{+} \underset{\text{LDH}}{\rightleftharpoons} \text{pyruvate} + NADH$$

The cuvette volumes and calculation of results were the same as for malic acid except that LDH was substituted for MDH.

The above three assays were taken from Bergemeyer, 1963

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