CHANGES IN GERMINATION PROMOTION AND INHIBITION IN SEED EXTRACTS OF SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.) RELATED TO DORMANCY AND GERMINATION

By M. G. WALKER*

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Abstract

Seeds of two varieties of *T. subterraneum* were extracted after imbibing water for various periods. The extracts were partitioned into water and ethyl acetate, purified by paper chromatography, and germination-modifying activity measured by a subterranean clover seed bioassay. As imbibition proceeded, the balance of germination inhibitors and promotors in the aqueous fraction remained fairly constant in the dormant variety, but changed rapidly in the non-dormant variety. The activity in the ethyl acetate fractions of both varieties also changed rapidly, but for the dormant variety the final balance was inhibitory, and in the non-dormant variety promotory.

After prolonged imbibition, germinated and ungerminated seeds were compared. Germination in the dormant variety was correlated with a decline in three water-soluble inhibitor zones, although it was concluded that only one was related to dormancy. One of these zones was absent in the non-dormant variety, but there was also an increase in a zone of inhibition apparently associated with anthocyanin pigments. Germination in both varieties was correlated with the appearance of germination promotors.

I. INTRODUCTION

The studies reported here were undertaken to test whether Amen's model of seed dormancy (Amen 1968), with its central concept of growth promotor–inhibitor balances as an endogenous control mechanism, could be related to germination and dormancy in subterranean clover (*Trifolium subterraneum* L.). Germination in this species appears to be controlled by two mechanisms, hard-seededness and embryo dormancy. Hard-seededness prevents water uptake by the seed, and may be broken by mechanical scarification or fluctuating temperatures (Quinlivan 1968). The embryo is said to be dormant if germination does not occur, even when the seed has imbibed (Morley 1961).

Recent studies on the endogenous control mechanism have not been conclusive. Ferguson (1967) added a crude extract from seed of a "rapid" germinating variety to

* Department of Agronomy, Institute of Agriculture, University of Western Australia, Nedlands, W.A. 6009.

seed of the same variety and temporarily reduced radicle growth. An extract from dormant seeds was not tested. Taylor and Rossiter (1967) found that washing excised dormant embryos accelerated germination. They concluded that this "supports the hypothesis that a water-soluble inhibitor is involved in the regulation of germination in subterranean clover". They further noted that purified seed extracts contained both promotory and inhibitory activity. However, this was measured by the wheat coleoptile straight growth bioassay.

These results provide only indirect evidence for Amen's model. The present study was undertaken to test the model directly.

II. Methods and Treatments

(*a*) *Extraction and Purification*

Samples comprising 200 seeds were macerated and extracted in 50 ml 80% ethanol for 5 hr. The extract was filtered and the filtrate evaporated to dryness under reduced pressure at 40°C. The residue was taken up in 10 ml distilled water and partitioned into four 10-ml aliquots of ethyl acetate. Both the ethyl acetate and the aqueous fractions thus produced were reduced to dryness, and the residues taken up in 1 ml acetate and water respectively. They were then strip-loaded on to sheets of Whatman 3 MM paper (46 by 57 cm).

The papers were developed in isopropanol–ammonia–water (10 : 1 : 1 v/v) for 25 cm, then air-dried overnight. The papers were then divided into the conventional 10 R_F fractions (each 2·5 cm wide) and then into 10 replicates per fraction (each 4·6 cm long), for the bioassay.

A blank of the complete procedure produced control papers.

(*b*) *Bioassay*

The method has been described in detail elsewhere (Walker 1971). Briefly, the paper fractions were assigned at random to plastic boxes (with tight-fitting lids) to which 0·9 ml distilled water were added, followed by 10 well-scarified subterranean clover seeds (cv. Woogenellup).

The 200 boxes thus prepared were incubated in the dark at 20°C for 20 hr and then the numbers of seeds per box with visible radicles were counted. Further counts were made at hourly intervals until the controls for each fraction had reached 50% germination. An analysis of variance was carried out on the difference between the 10 treatment–control pairs in which the controls were nearest to 50%. The results were plotted as the variance ratio between these differences and a pooled error term. The statistical design is such that over the range of activity normally encountered, this ratio is approximately linearly related to a treatment difference.

(*c*) *Treatments*

Extracts were made from scarified seed of the subterranean clover cultivars Woogenellup (non-dormant variety) and Clackline (dormant variety) after the following treatments and then assayed:

(1) Dry seed (0 hr imbibition), complete sample of 200 seeds.

(2) Seed after $\frac{1}{3}$ hr imbibition.

(3) Seed after 1 hr imbibition.

(4) Seed after 27 hr imbibition.

(5) Seed after 72 hr imbibition, 200 non-germinated seeds.

(6) Seed after 72 hr imbibition, 200 germinated seeds, radicle just emerged.

(7) Seed after 72 hr imbibition, 200 germinated seeds, radicle 10 mm long.

Ideally, the seeds for both varieties should have been allowed to imbibe for the same period in treatments (5)–(7). However, final germination percentage was so low in Clackline that with the limited seed available the test had to be extended to 144 hr to obtain sufficient germinated seed for extraction.

III. Results and Discussion

Assuming Amen's model to be relevant to subterranean clover seed germination and dormancy, the following predictions were made:

(1) The inhibitor–promotor balance would change in favour of promotors as imbibition and germination proceeded, the change being slower in the dormant variety.

(2) Germinated seeds would contain less than non-germinated seeds of any inhibitor causally related to dormancy.

(3) Germinated seeds would contain promotors causally related to the germinated state which would be absent in non-germinated seed.

(*a*) *Hypothesis 1*

This hypothesis should be verifiable from the data in Figure 1, which shows the activity in extracts after different imbibition periods. It can be seen that the balance in the aqueous fraction of the dormant variety remained inhibitory, there being two inhibitory zones and only one promotory zone. The picture is less clear for the ethyl acetate fraction. Two high R_F inhibitory zones in the dry seed disappeared after the imbibition period and one appeared in R_F zone 1. Further, two weak zones of promotion appeared. However, the overall balance was still inhibitory.

The activity in both the aqueous and ethyl acetate fractions of the non-dormant variety changed continually. In the aqueous fraction, inhibition at low R_F in the dry seed was replaced by apparently more significant activity at high R_F which had disappeared by the end of the imbibition period, the whole extract being inactive. Similarly, inhibition in R_F zone 4 of the ethyl acetate fraction was soon undetectable, and by the end, two promotory zones had appeared. Thus, the overall activity was promotory. On this evidence, the first hypothesis seems to hold.

(*b*) *Hypothesis 2*

Data to test this hypothesis is contained in Figure 2, which shows activity in extracts from either 100% germinated or non-germinated seeds. The hypothesis is partly verified for the dormant variety. Thus there were three distinct zones of inhibition in the aqueous fraction of extracts from non-germinated seeds, around R_F zones 1, 3, and 5–6. In the early stages of germination, R_F zone 1 was inactive and the activity in the other two zones markedly reduced. No ethyl acetate soluble inhibition was detected. It might, therefore, be anticipated that all three are involved in germination control. However, when the activity in well-germinated seeds is considered, it can be seen that the inhibitory activity in R_F zone 1 is on the threshold of significance, and, whilst the activity in R_F zone 3 is the same as in seeds just germinated, that for R_F zones 5–6 has resumed the level detected in the ungerminated seeds. The simplest explanation for the changes in zones 1 and 5–6 is that the inhibitor component remained constant during germination, the activity being masked by transient promotor activity. On this evidence the only inhibition causally related to dormancy appears to be that located in R_F zone 3. Further, the pattern is different when aqueous fractions of the non-dormant variety are considered. It can be seen

that the activity of R_F zone 1 was much greater both in the ungerminated and the germinated seeds, and when an extract was tested from seeds at a more advanced

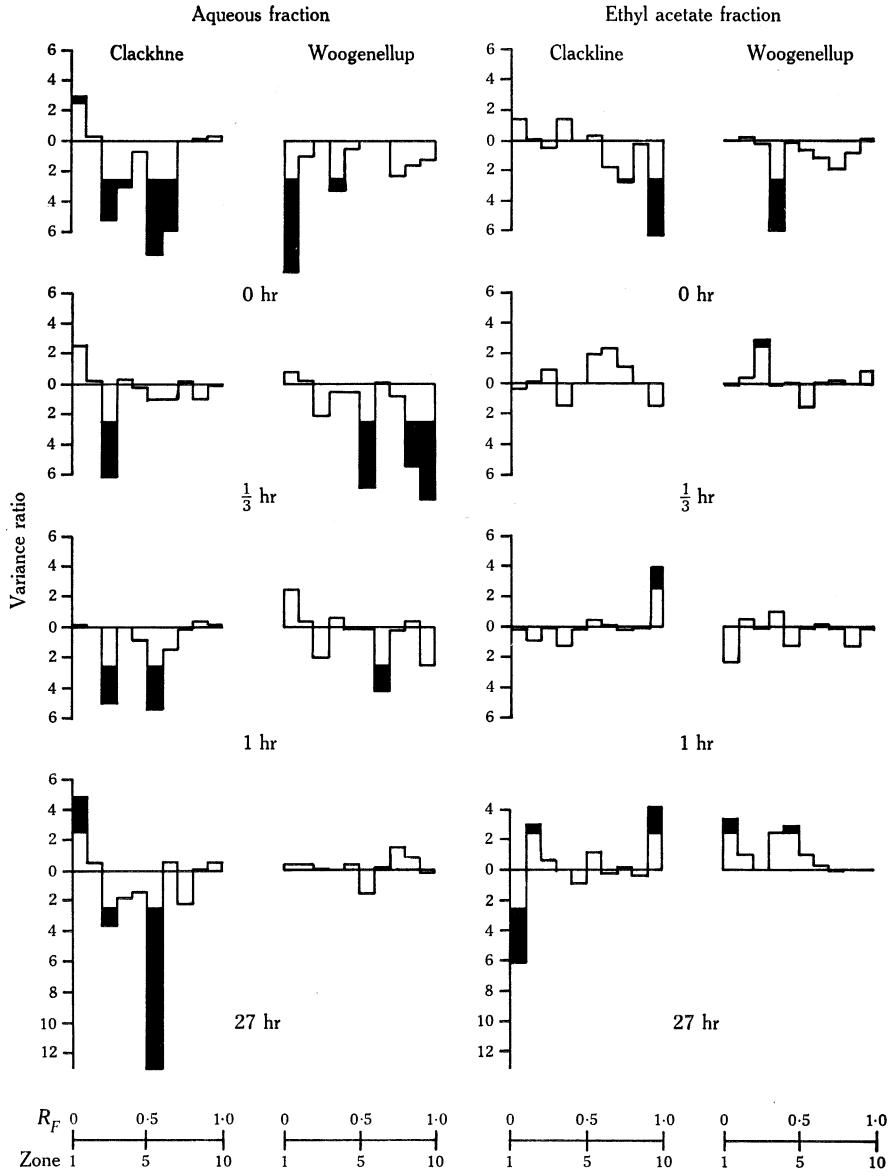

Fig. 1.—Comparison of germination-modifying activity in extracts made from the dormant (Clackline) and non-dormant (Woogenellup) varieties after various periods of imbibition. For each R_F zone, the activity is plotted as the variance ratio (for calculation, see text). Black areas indicate a significant effect, $P = 0{\cdot}01$.

stage of germination, this activity was even more marked. The loss of inhibitory activity in R_F zone 4 is similar but less marked than in R_F zone 3 of the dormant

variety. The apparent loss from R_F zone 9 appears to be due to the temporary superimposition of a promotor, since it reappears at a later stage of germination. It is, therefore, unlikely to be related to germination control.

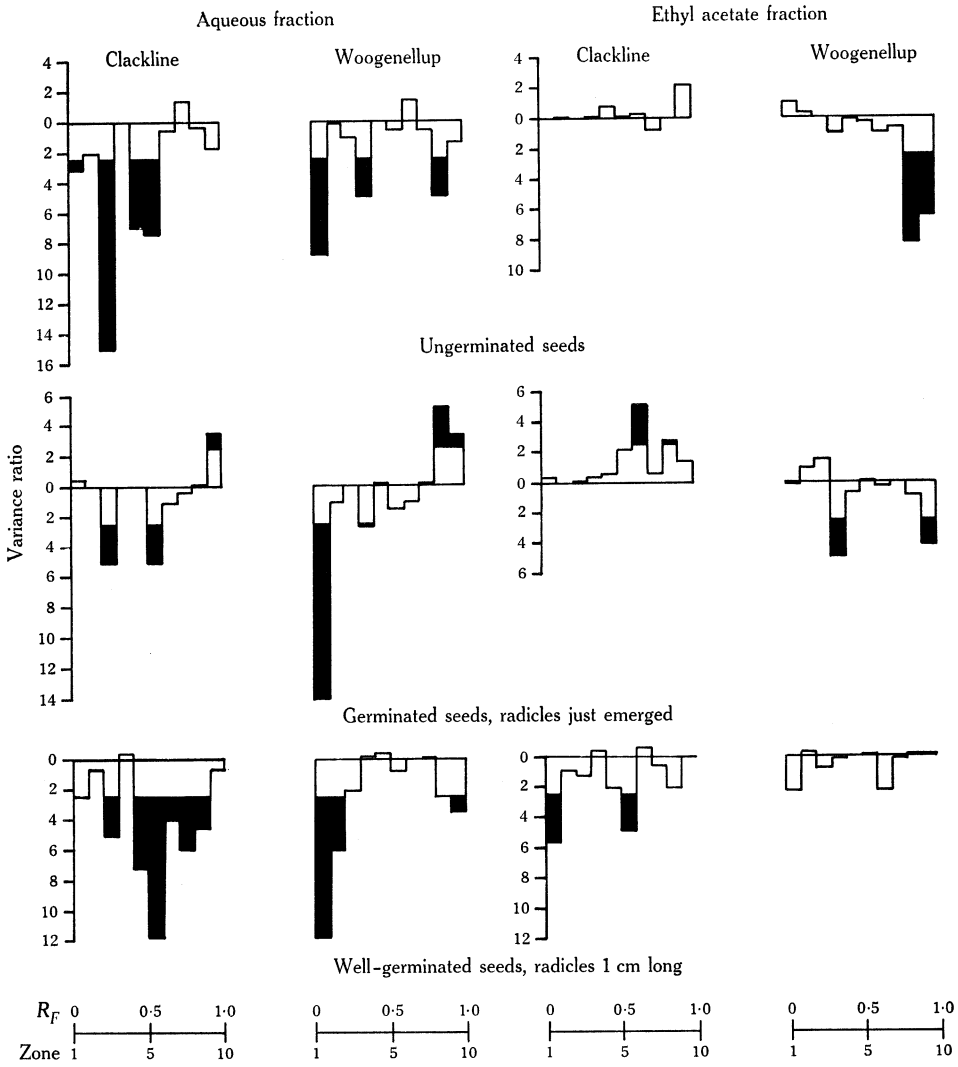

Fig. 2.—Comparison of germination modifying activity in extracts made from ungerminated seeds, seeds just germinated, and seeds with radicles 1 cm long. Details as for Figure 1.

When comparing the ethyl acetate fractions of the non-dormant variety, there appeared to be a zone of inhibition at high R_F which was not present in the dormant variety. This activity declined as germination proceeded. The transient appearance of inhibition at R_F zone 4 in the ethyl acetate fraction may have been due to incomplete partition of the activity detected in the aqueous fraction at this R_F. Such an

explanation would also preclude any role in dormancy control, since its activity declined *after* the emergence and growth of the radicle.

Thus, whilst the hypothesis appears to hold for the dormant variety, there are several anomalies to be explained in the non-dormant variety. The most obvious one is the marked inhibitory activity in R_F zone 1 of the aqueous extract. A similar response was detected by Frankland and Wareing (1966) in extracts of beech nuts but not hazel nuts. The activity detected here seemed to be related to pigments remaining near the chromatogram base line. These pigments changed colour with pH in a way characteristic of anthocyanins. Since the seed coats of the variety were purple, it was considered that these were the source of the pigment. It was observed that after germination tests the papers under the non-dormant variety were purple, due to pigment leaching, and those under the dormant variety were almost colourless, i.e. no pigment had been leached.

Assuming that the purple pigment was the source of inhibition, it was predicted that the non-dormant variety eluate would be inhibitory and the dormant eluate inactive. This was tested by soaking 200 seeds of each variety for 27 hr in 4 ml water, then making the eluate up to 10 ml and testing as described. The final germination percentage in the presence of the "non-dormant" eluate was 82% of the water control compared with 96% for the "dormant" eluate.

Carpenter (1961) has tentatively identified the seed coat pigments in subterranean clover as delphinidin glycosides. Stanton and Francis (1966) showed that anthocyanin glycosides comprise up to 5% of the seed coat of another legume, *Phaseolus vulgaris*, and that these compounds inhibited several species of bacteria, yeast, and fungi. The glycoside form of these pigments is more water-soluble than the aglycone. It was reasoned that hydrolysing the glycoside form of any inhibitor to the aglycone would reduce its elution and thus delay germination. Seeds were therefore germinated in the presence of 1% emulsin (a β-glycosidase). Germination was approximately 70% of the water controls for both varieties.

The precise function of the inhibition can only be speculative at this stage. Its probable location in the seed coat could suggest a function in controlling seed coat softening. This can occur by water uptake via the whole coat (Gladstones 1958; Quinlivan 1968) or via the strophiole (Hagon and Ballard 1970). If this softening is purely physical, then the removal of part of the seed coat would be the important factor, the fact that the part could also be inhibitory being coincidental. If it is considered a biochemical process, then the enzymic softening of the wall could well be controlled by an inhibitor. Thus Ikuma and Thimann (1963) demonstrated that kinetin promoted pectinase and cellulase softening of lettuce seed prior to penetration by the embryo, and Khan and Tolbert (1965) found that the kinetin promotion of this process could be reversed by coumarin. Further, gibberellins did not stimulate the system. The kinin benzyl adenine has been shown to stimulate subterranean clover germination, but gibberellic acid was shown to be ineffective (Walker 1971). More studies are obviously needed to clarify any interaction between embryo dormancy and hard-seededness.

(*c*) *Hypothesis 3*

This is clearly verified for the dormant variety. For extracts of germinated seeds, promotion was detected in R_F zone 10 of the aqueous fraction, and R_F zones 7

and 9 of the ethyl acetate fraction. There was no such activity in the ungerminated seeds. The aqueous R_F zone 10 promotor was also detected in the non-dormant variety. However, there was no activity in R_F zones 7 and 9 of the ethyl acetate fraction. This may have been because the sample was at a more advanced stage of germination than the dormant variety. This explanation is supported by the detection of two areas of promotion in the 27-hr extract of the non-dormant variety although the activity was in R_F zones 1 and 4.

Whether or not these zones of activity can be detected in both varieties when extracted at the same stage of germination remains to be seen.

IV. Conclusions

It is clear that the changes in the promotor–inhibitor balance in subterranean clover seeds prior to germination are complex. At present there is no data to warrant any attempt to fit the activity into the existing plant growth regulator classification. However, at least some of the activity detected can be taken as direct evidence for the relevance of Amen's model in this particular case, the evidence for which so far has been indirect.

V. Acknowledgments

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