KERNFORSCHUNGSZENTRUM

KARLSRUHE

November 1964 KFK 286

Institut für Strahlenbiologie

ESR Studies on Plant Seeds of Differential Radiosensitivity

I. Effect of Water Content

S. Bhaskaran

II. Effect of Oxygen and Nitric Oxide at Different Temperatures

S. Bhaskaran, W. Köhnlein

GESELLSCHAFT FUR KERNFORSCHUNG M. B. H.
KARLSRUHE

Radiation Botany, 1964, Vol. 4, pp. 285 to 289. Pergamon Press Ltd. Printed in Great Britain.

ESR STUDIES ON PLANT SEEDS OF DIFFERENTIAL RADIOSENSITIVITY—I. EFFECT OF WATER CONTENT

S. BHASKARAN
Institut für Strahlenbiologie, Kernforschungszentrum, Karlsruhe, Germany

(*Received* 17 *January* 1964)

Abstract—ESR studies on plant seeds of differential radiosensitivity were conducted with a view to evaluate the relationship between the production and decay of radicals and the observed differences in the radiosensitivity. Dry seeds (moisture content 5% and 2%) of *Agrostis*, tobacco and mustard were irradiated and the number of long lived radicals produced was determined. The yield of radicals in superdry seeds was much higher than in seeds of 5% moisture. At 5% level the yield of radical was least in mustard for a given dose as compared to tobacco and *Agrostis*. However, at 2% moisture content this relationship was altered in so far as mustard yielded the greatest number of radicals per gram for a given dose.

It is suggested that some substance, probably an oil-sugar complex, in mustard may be involved in the modification of the detectable concentration of radiation induced free radicals.

Résumé—Des études de la résonnance du spin de l'électron dans des graines de plantes possèdant différentes radio-sensibilité ont été réalisées en vue d'évaluer la relation entre la production et la décroissance des radicaux et les différences de radiosensibilité observées.

Des graines sèches d'*Agrostis* (humidité 5 % et 2 %), de tabac et de moutarde ont été irradiées et leur nombre de radicaux de vie longue produit a été déterminé. Le taux de radicaux dans des graines extrêmement sèches était beaucoup plus élevé que dans des graines à 5 % d'humidité. Au niveau 5 % et pour une dose donée, le taux de radicaux était moindre dans la moutarde comparativement au tabac et à *Agrostis*. Cependant pour une humidité de 2 % la relation était modifiée de telle manière que la moutarde contienne le nombre le plus élevé de radicaux par gramme pour une dose donée.

On suggère que chez la moutarde, certaines substances, probablement un complexe huile-sucre, peut être impliqué dans les modifications des radicaux libres induits par les radiations.

Zusammenfassung—An Pflanzensamen unterschiedlicher Strahlenempfindlichkeit wurden E.S.R.-Versuche durchgeführt mit dem Ziel, die Beziehung zwischen Auftreten und Zerfall freier Radikale sowie die beobachteten Unterschiede in der Strahlenempfindlichkeit zu klären. Trockene Samen (Wassergehalt 5 % und 2 %) von *Agrostis*, Taback und Senf wurden bestrahlt und die Anzahl der entstandenen langlebigen Radikale bestimmt. Die Radikalausbeute war in sehr trockenen Samen viel höher als in jenen mit 5 % Feuchtigkeit. Ein Vergleich zeigte, dass bei 5 % Feuchtigkeit die Radikalausbeute bei einer gegebenen Strahlendosis in Senf geringer war als bei Taback oder *Agrostis*. Bei 2 % Feuchtigkeit war dieses Verhältnis jedoch insofern verändert, als Senf bei gegebener Strahlendosis die grösste Zahl freier Radikale pro Gramm aufwies.

Man vermutet, dass eine bestimmte Substanz im Senf, eventuell ein Öl-Zucker-Komplex, bei der Veränderung der nachweisbaren Konzentration der strahleninduzierten freien Radikale eine Rolle spielen könnte.

INTRODUCTION

THERE is considerable evidence for assuming that long lived free radicals produced in irradiated biological systems play a significant role in the manifestation of biological damage. Electron spin resonance (ESR) spectroscopy provides a sensitive means of detecting and measuring these free radicals. A number of investigators have been exploiting this technique for the study of the relationship between free radical production and decay and the observed biological damage since 1957, when ZIMMER *et al.*[(10)] first reported radiation induced free radicals in plant seeds. Many of the experimental results accumulated so far show remarkable similarities between the effect of various environmental and storage conditions on the production and decay of radicals and on the after effect in seeds.[(4,5,9)]

Plant seeds are characterized by their extreme differences in radiosensitivity. The present studies were initiated with a view to inquire into the production of long lived free radicals in seeds of differential radiosensitivity and thus form an attempt to explain the observed differences in radiosensitivity among plant seeds. Seeds of *Agrostis stolonifera*, *Nicotiana tabaccum* (tobacco), and *Brassica campestris* (mustard), representing three levels of radiosensitivity, were selected for this purpose. The LD_{50} (mean lethal doses) for these three types of plant seeds are about 20 kr, 70 kr and 150 kr respectively.

MATERIAL AND METHODS

Seeds of *Agrostis*, tobacco and mustard were equilibrated to two levels of moisture content (5% and 2%). The seeds were kept in a desiccator containing drying agents like $CaCl_2$ and P_2O_5. The moisture level of 2% was attained by drying the seeds over P_2O_5 under a very high vacuum for about 8 weeks. Irradiations and ESR measurements were performed with whole seeds and no attempt was made to separate the different components. Dose effect curves were obtained by using hard X-rays (150 kV, 25 mA, H.V.L. of 6 mm Al, effective wavelength 0·26 Å) at a dose rate of 3000 r/min. The radiation dose was measured by three independent methods and was found to be accurate within 5%[(7)]. All irradiations and ESR measurements were made at room temperature and in air. A commercial ESR spectrometer (Varian Associates) with double sample cavity was used for the measurements. The details of the procedure have been reported in earlier papers[(6)]. Evaluations of the first derivatives of absorption spectra were performed with a momentum balance.[(7,11)]

OBSERVATIONS

Unirradiated seeds of *Agrostis*, tobacco and mustard gave rise to signals. Dose effect curves constructed after subtracting the background concentration of free radicals are given in Figs. 1 and 2. The dose effect curves were linear for

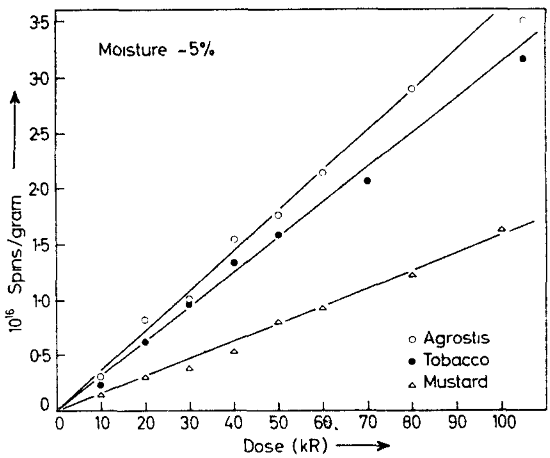

FIG. 1. Dose effect curves of *Agrostis stolonifera*, tobacco and mustard of 5% moisture content.

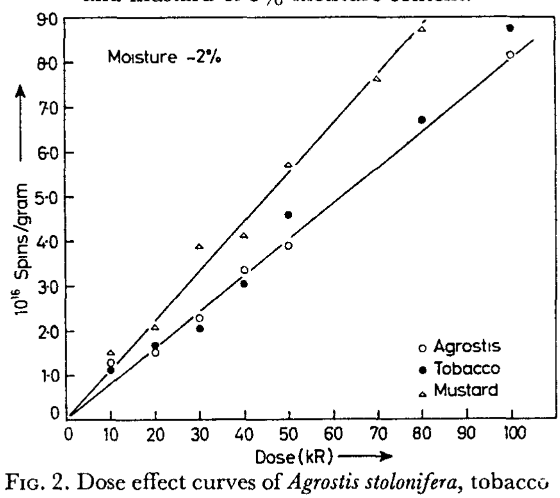

FIG. 2. Dose effect curves of *Agrostis stolonifera*, tobacco and mustard of 2% moisture content.

all the three types of seeds up to the maximum dose given (100 kr). All the three types of seeds on irradiation gave rise to a non-specific signal, which, no doubt, is an envelope of different species of radicals produced. At 5% moisture level (Fig. 1) the free radical concentration per g of seed showed a difference among the seeds used. *Agrostis* seeds yielded maximum and mustard the minimum number of radicals per gram per dose. The concentration of radicals in tobacco was a little less than in *Agrostis*. This observation, taken alone, is in conformity with the differential radiosensitivity of the three types of seeds. From Fig. 2 it may be seen that the super-dry seeds (2%) yielded more radicals per gram as compared to the yield at 5% moisture level. Furthermore, the parallelism observed between the concentration of free radicals and differential radiosensitivity at 5% moisture level was not found in seeds with 2% moisture. *Agrostis* and tobacco yielded the same number of radicals and mustard gave rise to slightly more than the other two types of seeds. It was thought that these differences in the relationship between the radical yield and moisture content in the three types of seeds investigated could be due, either to dried melanin pigment, present in the seed coat of mustard seeds, and yielding a greater number of radicals on irradiation, or to some substances, probably oils, escaping from the seeds and acting as radical scavengers when present in the material. To test these hypotheses, seeds with 2% moisture were rehydrated to 5% and the radical yield was measured. The results are presented in Fig. 3. It is seen from Fig. 3 that on rehydration to 5% the radical yield in mustard was greater than in tobacco and *Agrostis*, whereas the radical yield in tobacco and *Agrostis* reached more or less the same value as in the seeds of original moisture content of 5%. To test further whether the oils as such play any role in modifying the radical yield, mustard seeds of 2% moisture were soaked in mustard oil, and ESR measurements were made after irradiation. The radical yield did not show any change by this treatment showing that oil as such does not act as radical scavenger.

From the radical concentrations measured and the doses applied the yields expressed as the number of radicals per 100 eV of absorbed energy (i.e., as G-values) were calculated for the seeds irradiated at different moisture levels and are given in Table 1. To study the decay of radicals, ESR measurements were made at different time intervals after irradiation. Radical decay curves showed two components: an initial decay which was more rapid and covered a period of 15 hr, and a second component which decayed at a slower rate. The rate of decay was the same in tobacco and *Agrostis* whereas the decay in mustard was more rapid. About 200 hr after irradiation there was no appreciable further decay of radicals.

Table 1. Number of radicals detected per 100 eV *of absorbed energy in* Agrostis, *tobacoo and mustard irradiated at two different moisture levels*

Material	Moisture content 5%	2%
Agrostis stolonifera	0·71	1·55
Tobacco	0·65	1·55
Mustard	0·31	2·10

DISCUSSION

The results presented above reveal that seeds of differential radiosensitivity do show a difference in the yield of radicals when the seeds are equilibrated to a moisture content of 5%. However, when the seeds were superdried to 2% over P_2O_5 in high vacuum and for a long time, the relationship between the concentration of radicals and differential radiosensitivity was no longer valid, in so far as mustard now gave rise to a greater number of radicals than the other two types of seeds though the concentration of the radicals was increased in all three species. This phenomenon has been observed repeatedly in plant material.[2,4] Super-dry seeds are extremely radiosensitive and this feature is often explained by considering that free radicals produced in this condition have a smaller chance of mobility than in the seeds with higher moisture and consequently do not recombine with each other.[2,4]

The yield of radicals in *Agrostis* and in tobacco at 2% moisture was about twice that at 5%, whereas in mustard the factor of increase was about 7. Thus mustard seeds, though at 5% moisture level yielding the smallest number of radicals as compared to *Agrostis* and tobacco, at 2% moisture level give a radical yield per gram higher than in the two other kinds of seeds. This increase in the yield of radicals in super-dry mustard seeds could be due, as mentioned above, either to the melanin pigment present on the seed coat assuming that on irradiation the dried melanin yields a greater number of radicals, thus increasing the total yield per gram of seed. Alternatively the increased radical production might be due to the removal of some substance, probably an oil complex, from the seeds by drying under vacuum and this substance might be acting as a radical scavenger when present. The results presented in Fig. 3 show that melanin cannot be responsible for the observed increase in radical yield, since the super-dry mustard seeds on rehydrating to 5% show a radical yield higher than in the normal seeds with 5% moisture. *Agrostis* and tobacco

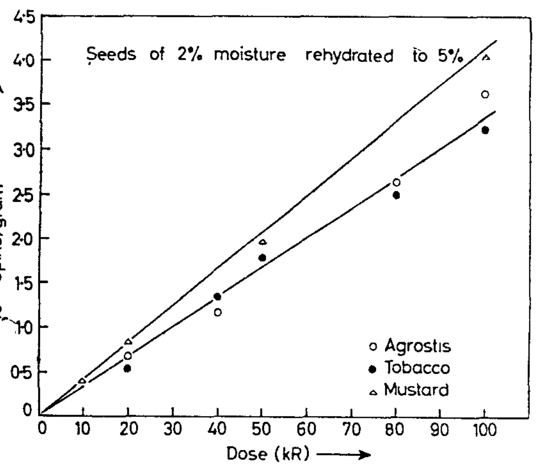

FIG. 3. Dose effect curves of *Agrostis stolonifera*, tobacco and mustard, seeds of 2% moisture rehydrated to 5%.

seeds of 2% moisture, however, on rehydrating to 5% yielded more or less the same as in the original seeds with 5% moisture. Thus this result indicates that some component in the mustard seed escapes or is destroyed during drying under a very high vacuum. Though we have not been able to identify the substance or substances escaping from the seeds, it was thought that oils are the most likely ones to escape. Super-dry seeds soaked in mustard oils, however, did not show any appreciable decrease in the yield, thus suggesting that oils as such are not likely to be responsible for the modification of the radical yield. In mustard seeds, oils occur in the form of a complex with sugar and it is possible that this complex is more important than oils alone. It is, however, noteworthy that irradiated oils do not yield any radicals.[2] The data of SINGH *et al.*[8] working with two different strains of maize, low oil content and high oil content, show that the amount of detectable free radicals in the low oil content strain was higher than that in the high oil content strain. They further observed that the decay of radicals in high oil content strain was faster than in low oil content strain, which is in good agreement with our results, and they have attributed this behaviour of the high oil content strain to the greater mobility of the free radicals in the medium. From the present studies we cannot, therefore, exclude the possibility that substances other than oil play a role in the modification of radical yield in mustard seed.

Whatever may be the nature of the substance or substances involved in modifying the radical yield of mustard it can be concluded from our present results that the extent of secondary processes like recombination and scavenging of radicals is of preponderant importance in determining the final concentration of radicals measured. ESR studies on *Agrostis*, tobacco and mustard treated with O_2 or NO and irradiated at low temperatures (110°K) have yielded results which support this view.[1]

Acknowledgements—I wish to express my thanks to Prof. K. G. ZIMMER for his interest and encouragement. I am thankful to Drs. W. KÖHNLEIN and A. MÜLLER for their advice and helpful discussions.

The financial assistance of Alexander von Humboldt-Stiftung which made this study possible, is gratefully acknowledged.

REFERENCES

1. BHASKARAN S. and KÖHNLEIN W. (1964) ESR studies on plant seeds of differential radio-

sensitivity II. Effect of oxygen and nitric oxide at different temperatures. (In press).

2. CONGER A. D. and RANDOLPH M. L. (1959) Magnetic centers (free radicals) produced in cereal embryos by ionizing radiation. *Radiation Research* **11**, 54–66.

3. EHRENBERG A. (1961) Research on free radicals in enzyme chemistry and in radiation biology, pp. 337–350. In: BLOIS *et al.* (eds.), *Free radicals in biological systems*. Symposium held at Stanford University, 1960.

4. EHRENBERG A. and EHRENBERG L. (1958) The decay of X-ray induced free radicals in plant seeds and starch. *Arkiv Fysik* **14**, 133–141.

5. EHRENBERG A., EHRENBERG L. and LÖFROTH G. (1962) Radiation induced paramagnetic centers in plant seeds at different oxygen concentrations. *Abhandl. deut. Akad. Wiss, Berlin. Kl. Med.* **1**, 229–231.

6. KÖHNLEIN W. and MÜLLER A. (1960) Ein Doppelhohlraum für Präzisionsmessungen der Konzentration freier Radikale. *Z. Naturforsch.* **15b**, 138–139.

7. KÖHNLEIN W. and MÜLLER A. (1962) Absolute yield measurements of radiation produced radicals by electron spin resonance. *Phys. Med. Biol.* **6**, 599–604.

8. SINGH B. B., VENKATERAMAN B., NOTANI N. K., CHANDRA MOULI and BORA K. C. (1962) Role of chemical composition of biological material on the production and decay of radiation induced free radicals and their identification. pp. 3–13. *Symposium on "Biological effects of ionizing radiation at the molecular level"*, IAEA. Vienna, 1961.

9. SPARRMAN B., EHRENBERG L. and EHRENBERG A. (1959) Scavenging of free radicals and radiation protection by nitric oxide in plant seeds. *Acta Chem. Scand.* **13**, 199–200.

10. ZIMMER K. G., EHRENBERG A. and EHRENBERG L. (1957) Nachweis langlebiger magnetischer Zentren in bestrahlten biologischen Medien und deren Bedeutung für die Strahlenbiologie. *Strahlentherapie* **103**, 3–15.

11. ZIMMER K. G., KÖHNLEIN W., HOTZ G. and MÜLLER A. (1963) Elektron-Spin-Resonanzen in bestrahlten Bakteriophagen und deren Bestandteilen. I. Mitteilung. *Strahlentherapie* **120**, 161–190.

Radiation Botany, 1964, Vol. 4, pp. 291 to 298. Pergamon Press Ltd. Printed in Great Britain.

ESR STUDIES ON PLANT SEEDS OF DIFFERENTIAL RADIOSENSITIVITY—II. EFFECT OF OXYGEN AND NITRIC OXIDE AT DIFFERENT TEMPERATURES

S. BHASKARAN* and W. KÖHNLEIN†

Institut fur Strahlenbiologie, Kernforschungszentrum, Karlsruhe, Germany

(*Received* 17 *January* 1964)

Abstract—Dry seeds of *Agrostis*, tobacco, and mustard, treated at 1 atm pressure with oxygen, nitric oxide, and heat were irradiated with X-rays and free radical concentration was measured by the ESR-method. It was found that NO treatment for 110 hr decreases the radical yield, whereas heat-pretreatment did not affect the number of radiation induced radicals in comparison with seeds treated with O_2. From the shape of the resonance spectra at least two radical components were evident. The ratio of these two components varies with the treatment prior to irradiation. A possible explanation for the protective effect by NO and heat-pretreatment in seeds is given.

Résumé—Des graines sèches d'*Agrostis*, de tabac et de moutarde ont été traitées par de l'oxygène, de l'oxide nitrique et par la chaleur puis irradiées par les rayons X. La concentration en radicaux libres y a été mesurée par la méthode de la résonnance du spin de l'électron. On a montré qu'un traitement par NO pendant 110 heures diminue la concentration des radicaux alors qu'un pré-traitement par la chaleur n'affecte pas le nombre de radicaux induits par les radiations comparativement à des graines traitées par O_2. D'après la forme des spectres de résonnance, on a démontré l'existence d'au moins 2 genres de radicaux. Le rapport de ces 2 composantes varie avec la nature dù traitement précédant l'irradiation. On fournit une explication possible de l'effet protecteur de NO et de pré-traitement par la chaleur.

Zusammenfassung—*Mit Hilfe der Elektronenspinresonanz-Methode wurden die strahleninduzierten freien Radikale in* *Agrostis*-, Tabak- und Senfsamen untersucht. Vor der Bestrahlung wurden die Samen mit Sauerstoff, Stickoxyd bzw. mit einer Wärmebehandlung vorbehandelt. Während eine Stickoxydbehandlung die Radikalausbeute verringert. beeinflusst eine Wärmebehandlung nicht die Anzahl der strahleninduzierten freien Radikale verglichen mit der Radikalausbeute nach einer Sauerstoffbehandlung. Bei der Bestrahlung werden mindestens zwei verschiedene Radikalarten gebildet. Das Verhältnis dieser beiden Komponenten ist von der Behandlung der Samen vor und nach der Bestrahlung abhängig. Eine mögliche Erklärung für den Schutzeffekt durch Stickoxyd und durch Wärmebehandlung in den Samen wird diskutiert.

INTRODUCTION

MUCH evidence has already been accumulated to show that free radicals frequently play a significant role in the chain of events following the primary physical process of energy absorption in biological material.[11] ESR observations on plant seeds have yielded results which might explain some of the effects of radiation such as modification of the radiation damage by oxygen concentration, water content and temperature (for literature see ZIMMER *et al.*[12]). The nature of the free radicals involved is, however, not

* Present Address: Division of Botany, Indian Agricultural Research Institute, New Delhi-12, India.

† Present Address: Department of Molecular Biology and Biophysics, Yale University, 2166 Yale Station, New Haven, Connecticut, U.S.A.

known. Recently, EHRENBERG *et al.*[5], irradiating dry seeds under different conditions, have shown the possibility of classification of the types of radicals produced depending on their reaction with oxygen.

The present ESR studies on *Agrostis stolonifera*, *Nicotiana tabaccum* (Tobacco) and *Brassica campestris* (mustard) seeds representing three levels of radiosensitivity, are an attempt to gain information on radical yield and on the types of radicals produced by irradiation under different conditions, such as presence of oxygen and nitric oxide and varying temperatures. Nitric oxide and heat treatment prior to irradiation is known to bring about protection in plant material.[2,4,9,10] Thus it should be interesting to investigate whether ESR measurements could throw light on the mechanism of protection against radiation in plant material treated with nitric oxide or heat.

MATERIAL AND METHODS

Dry seeds of *Agrostis*, tobacco, and mustard, of moisture content of about 4 per cent, were used for the present studies. The average weights per seed of *Agrostis*, tobacco and mustard are 0·08, 0·1, and 3·5 mg respectively. The nitric oxide was obtained as 99 per cent pure gas from Matheson Co., Inc., East Rutherford, N.J., U.S.A. Possible contaminants of the nitric oxide, such as NO_2 and N_2O_3 which are very soluble in water, were removed by bubbling the gas in very tiny bubbles through water. Remaining contaminants were assumed to be eliminated in a cooling trap following the water treatment. The temperature of the trap was kept at −78°C. Irradiations were performed at room (295°K) and at low (110°K) temperatures. For the oxygen experiments the seeds were flushed prior to irradiation with O_2 at a pressure of 1 atm for about 25 hr. Considering the fact that very small seeds were used, this time seems to be sufficient to saturate them with O_2[8]. For nitric oxide experiments, purified NO was admitted to the tubes containing seeds which had previously been evacuated on a high vacuum line for about 15 hr. After incubation times of 4 and 110 hr at a pressure of 1 atm the seeds were irradiated in the presence of NO. The ESR measurements were also performed in the presence of NO. Measurements were also made with *Agrostis* seeds which were given a pretreatment of heat (100°C for 1 hr *in vacuo*). These seeds were irradiated and measured also *in vacuo*.

The material was irradiated with X-rays (150 kV, 20 mA, H.V.L. 6 mm Al, effective wave length 0·26Å) at a dose rate of about 1 kr/min. Only that region of the tube containing the sample was irradiated. The unirradiated part of the tube was used for ESR measurements after the seeds had been shaken to the other end. For the irradiation at low temperatures (110°K) the quartz tubes were flushed by a stream of precooled nitrogen gas.

A commercial X-band ESR-spectrometer from Hilger and Watts (England) with 100 kc field modulation and first derivative presentation was used for the detection of radiation induced radicals. For quantitative measurements of radical concentration we used a double sample cavity described previously[6]. The equipment was connected with a variable temperature accessory for the temperature range from about 100°K to 300°K. To avoid microwave power saturation, measurements were carried out at various power levels. It appeared that especially at low temperature (110°K) power saturation was already appreciable at 0·2 mW. Evaluations of the first derivatives of absorption spectra were performed with a planimeter delivering the first moment proportional to the spin concentration[7].

RESULTS

Dose effect curves for *Agrostis*, tobacco, and mustard seeds treated with O_2 (24 hr) and NO (4 hr and 110 hr) at room (295°K) and low temperatures (110°K) are given in Figs. 1–3. In all the cases the dose effect curves were linear up to 315 kr, the highest dose used. At room temperature, the seeds treated with oxygen or with nitric oxide for 4 hr and irradiated and measured in these atmospheres yielded the same number of radicals per gram. Further, it was found that mustard seeds yielded a smaller number of radicals as compared to *Agrostis* and to tobacco seeds at room temperature. Seeds incubated in nitric oxide for 110 hr prior to irradiation, and irradiated and measured in the presence of NO at room temperature showed a reduction in the radical yield in comparison with seeds treated with oxygen or nitric oxide for only 4 hr.

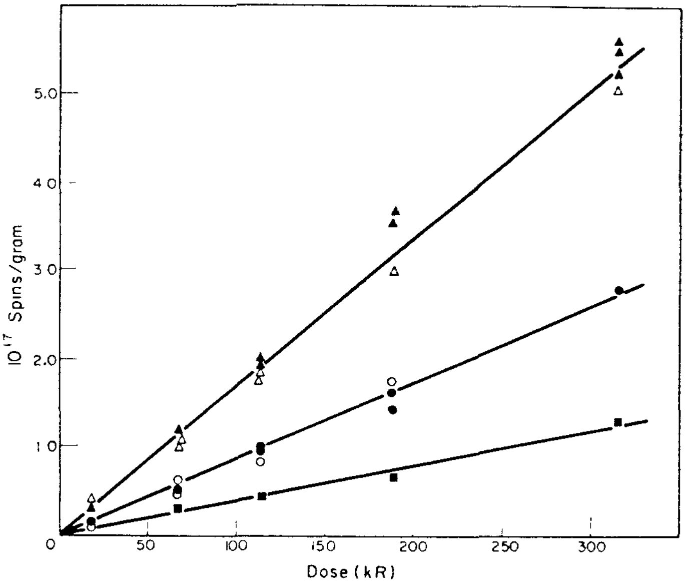

FIG. 1. Dose effect curves for radiation induced free radicals in *Agrostis stolonifera* under various conditions.

(●): pretreated with NO for 4 hr at 1 atm and 295° K, irradiated and measured in the presence of NO at 295°K.

(■): pretreated with NO for 110 hr at 1 atm and 295°K, irradiated and measured in the presence of NO at 295°K.

(▲): pretreated with NO for 4 hr at 1 atm and 295°K, irradiated and measured in the presence of NO at 110°K.

(○): pretreated with O_2 for 25 hr at 1 atm and 295°K, irradiated and measured in the presence of O_2 at 295°K.

(△): pretreated with O_2 for 25 hr at 1 atm and 295°K, irradiated and measured in the presence of O_2 at 110°K.

Reduction in the yield of free radicals in *Agrostis* and in tobacco was about 50 per cent, and in mustard about 40 per cent, as compared to the concentration at room temperature in the presence of oxygen. All the three types of seeds treated with O_2 or NO at 295°K but irradiated and measured at low temperature (110°K) in the presence of O_2 and NO respectively gave 2 to 2·5 times the yield at room temperature; the radical concentration was the same irrespective of treatment (oxygen or nitric oxide).

From the dose effect curves the yields were calculated as the number of radicals per 100 eV of absorbed energy, i.e. as G-values, which fall in range of about 1 to 2. The results are given in Table 1. At room temperature, mustard appears to be less sensitive than tobacco and *Agrostis*, whereas at low temperature (110°K) all the

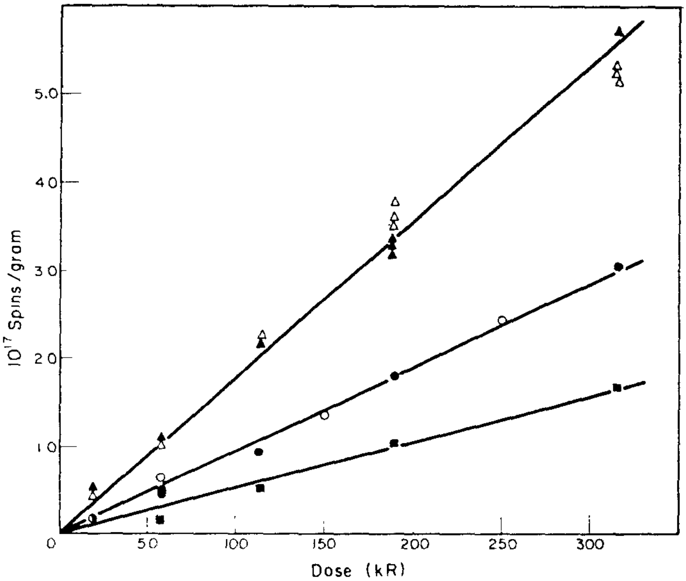

FIG. 2. Dose effect curves for radiation induced free radicals in tobacco seeds under various conditions. The symbols and conditions described as in Fig. 1.

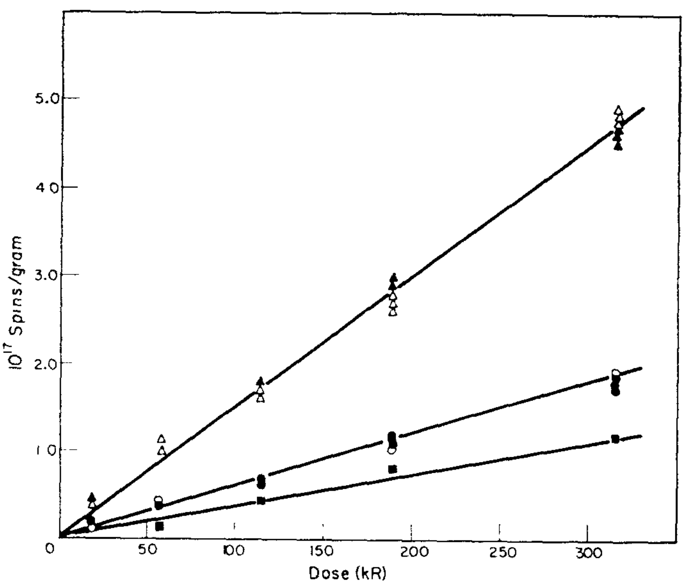

FIG. 3. Dose effect curves for radiation induced free radicals in mustard seeds under various conditions. The symbols and conditions as described in Fig. 1.

Table 1. *Number of radicals detected per* 100 *eV of energy absorbed in seeds under various circumambient conditions*

Material	295°K Oxygen or nitric oxide (4 hr)	295°K Nitric oxide (110 hr)	Vacuum and heat treatment	110°K Oxygen or nitric oxide (4 hr)
Agrostis	1·7	0·75	2·5	3·3
Tobacco	1·8	1·0	—	3·5
Mustard	1·1	0·75	—	3·1

three species showed equal sensitivity.

The decay rates as measured by the amplitude and radical concentration showed two components: the first one had a faster rate of decay and covered a period of 15 hr; the second component had a slower rate of decay. The rate of decay in mustard seeds was a little faster than in *Agrostis* and tobacco seeds. Thus, 150 hr after irradiation, the concentration of radicals in *Agrostis*, tobacco and mustard was 70, 70 and 55 per cent, respectively, of the original concentration. At low temperature (110°K), seeds treated with oxygen and nitric oxide showed decay in radical concentration, when the measurements were made at various temperatures from 110°K to 295°K. As the seeds gradually warmed up to room temperature, the radical concentration fell to about 50 per cent of the original concentration in *Agrostis* and in tobacco, and to about 35 per cent in mustard, whereas the amplitudes of the signals remained almost constant. Consequently, at least two radical components are involved (Fig. 4).

The ESR signals obtained at lower doses in seeds treated with oxygen and nitric oxide at 295°K and 110°K were narrow and nonspecific,

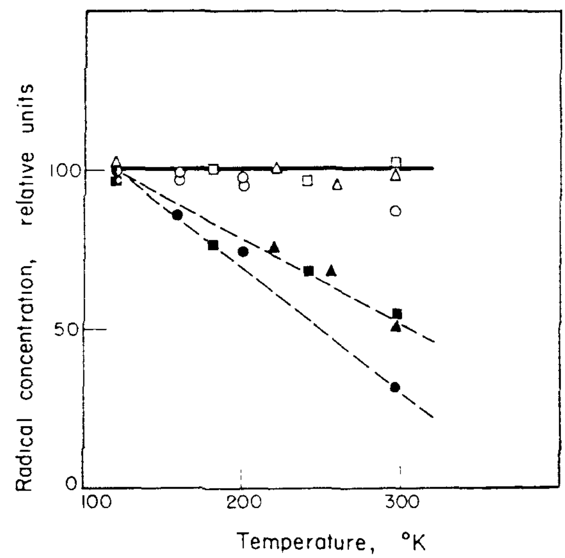

FIG. 4. Decay of radical concentrations and amplitudes of first derivatives of absorption spectra of *Agrostis stolonifera*, tobacco, and mustard on slowly warming up to 300°K after irradiation at 110°K.

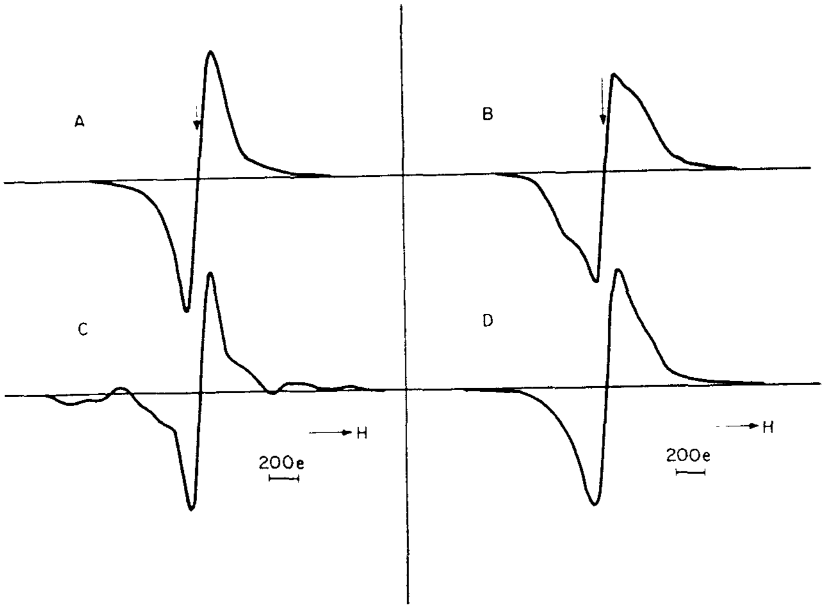

FIG. 5. First derivatives of absorption spectra of *Agrostis stolonifera*, tobacco, and mustard after different treatments. Arrows pointing downward represent the position for DPPH resonance (g = 2,0036). See text for more details.

with a linewidth of about 7–9 Oersted from peak to peak and without any hyperfine structure (Fig. 5A). At higher doses, however, the signals became broader and in the case of *Agrostis* seeds treated with nitric oxide a clear superimposed signal appeared with a linewidth of about 20 Oersted from peak to peak (Fig. 5B). At higher doses, tobacco seeds treated with nitric oxide for 110 hr gave rise to one more side line of small intensity which was about 30 Oersted away from the main line (Fig. 5C). *Agrostis* seeds evacuated under a high vacuum line for about 15 hr and irradiated *in vacuo* also gave rise to the superimposed signal at higher doses (Fig. 5B). A similar signal was also obtained when a pretreatment of heat (100°C for 1 hr) was given. The superimposed signal remained stable *in vacuo*, but when oxygen was admitted to the tube the superimposed signal decayed within about 24 hr and a narrow non-specific signal was obtained (Fig. 5D). Measurements made 120 hr after irradiation of seeds under vacuum still showed the superimposed signal. The decay of radicals under vacuum and in air showed a clearcut difference: the decay under vacuum was quite negligible even 144 hr after irradiation, whereas radicals in seeds irradiated *in vacuo* and then exposed to air decayed rapidly in the beginning and slowly afterwards. The concentration of radicals 144 hr after irradiation was 35 per cent of the original concentration (Fig. 6).

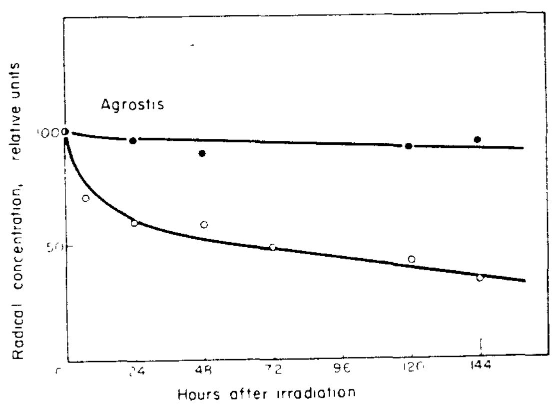

FIG. 6. Decay of radical concentration in *Agrostis stolonifera* irradiated *in vacuo* and stored *in vacuo* (•) or in air (○).

The experimental findings may be summarized as follows: (1) among the seeds treated with oxygen, mustard yielded the smallest number of radicals per gram at room temperature, tobacco next and *Agrostis* most; (2) nitric oxide treatment for 110 hr decreased the radical yield of all three species; (3) irradiations at low temperature (110°K) increased the radical concentration by a factor of 2 to 2·5; (4) when the seeds irradiated at low temperature (110°K) were gradually warmed up to 300°K, the concentration of radicals decayed to almost the same concentration as obtained after irradiation at room temperature; (5) when *Agrostis* seeds were evacuated for 15 hr or given a pretreatment of heat (100°C for 1 hr), a broad superimposed signal was obtained which remained stable under vacuum but decayed rapidly on exposure to air.

DISCUSSION

The present investigations indicate that, apart from quantitative differences in yield, the shape of the ESR signal obtained from irradiated seeds is influenced by the surrounding gas, especially by the presence or absence of oxygen. Similar observations have been reported by EHRENBERG *et al.*[5]

Measurements at room temperature of seeds treated with nitric oxide for 4 hr did not show any difference in the radical yield as compared to seeds treated with oxygen. Mustard seeds, at room temperature, gave rise to a smaller number of radicals than tobacco and *Agrostis* seeds, thus confirming the results obtained at a 5 per cent moisture level[1]. Because of its single unpaired electron, nitric oxide in reacting with a free radical should form an unreactive species. The reaction product terminates the activity and thus a reduction in the concentration of radicals is to be expected. The observation that reduction in radical yield was obtained only in seeds treated for 110 hr indicates that 4 hr treatment may not be sufficient for diffusion of nitric oxide into the seeds. Thus, to observe the protective action of nitric oxide in biological material like plant seeds, pretreatment for a sufficient period, in the present case longer than 4 hr, must be given. Nitric oxide, though possessing a magnetic moment, does not interfere with our ESR measurements, since the magnetic resonance spectrum of NO observed at a frequency of 9360 Mc appears at a magnetic field strength of 8600 Oersted[3] whereas the present measurements were made at a field strength of 3300 Oersted.

Seeds irradiated and measured at 110°K gave rise to a higher yield of radicals, by a factor of 2 to 2·5 compared to the yield at 300°K. The increased radical yield could be due to excluding the role of water in facilitating recombination of radicals, which otherwise takes place at higher temperatures (300°K). Another observation supports this view: the radical yield, in three species used, is increased when the moisture content is decreased from 5 per cent to 2 per cent[1]. Further, it was found that when the seeds irradiated at 110°K were gradually warmed up to 300°K the concentration of radicals was about the same as in seeds irradiated at 300°K. It is also significant that mustard, which gave rise to a smaller number of radicals at 300°K than *Agrostis* and tobacco, yielded almost the same number of radicals at 110°K as the other two species (Table 1). This suggests that seeds of differential radiosensitivity are potentially capable of yielding the same number of radicals when the secondary processes like radical recombination and reaction with water are excluded. In other words, the magnitude of the secondary processes determines the radical yield in seeds of differential radiosensitivity.

Turning to the qualitative results, we obtained a nonspecific narrow signal when the seeds were irradiated in the presence of oxygen. However, at higher doses ($>$100 kR) the signal became broader. When the seeds were evacuated for about 18 hr or a pretreatment of heat for 1 hr at 100°C *in vacuo* was given, the broad superimposed signal appeared at lower doses. The superimposed signal was stable in vacuum even 150 hr after irradiation, but on exposure to air it decayed very rapidly and 24 hr after the vial was opened only a narrow signal was obtained. EHRENBERG *et al.*[5] have also obtained broad signals at higher doses and when pretreatment of heat was given to seeds. They suggested that the radicals causing the narrow signal are of a special chemical type and less sensitive to oxygen than the radicals causing the broader signal, which begins to appear when the intracellular oxygen has been consumed. The present studies with seeds of three species of plants confirm this hypothesis. At a given dose, *Agrostis* gave rise to the most pronounced broad signal, whereas in tobacco and mustard the broad signal was less marked. This decrease in the ratio between the narrow and broad signals in different materials may be governed by the amount and the rate of removal of intracellular oxygen, which might depend upon the relative permeability of the seed coat.

The protective effect of heat-pretreatment in plant seeds(2,4) could be explained, as EHRENBERG *et al.*(5) have suggested, on the basis of the removal of O_2 from the seeds, since after a heat-pretreatment irradiation induces less of the narrow signal radicals, although the total number of radicals was not reduced. In the present studies, too, we found no reduction in free radical concentration in seeds given heat-pretreatment. From these observations it would appear that the radicals giving broad signals may be relatively less harmful in producing radiobiological damage. Thus the mechanism of protection in seeds by nitric oxide and heat-treatments seems to be different. Nitric oxide protects the seeds by removing the free radicals formed, whereas protection by heat-treatment may be due to the removal of oxygen, thus preventing the formation of the harmful radicals which give rise to the narrow signal.

Acknowledgements—We wish to express our thanks to PROF. K. G. ZIMMER for his interest and encouragement.

One of us (S.B.) is grateful to the Alexander von Humboldt-Stiftung for the award of a fellowship, which made this study possible.

REFERENCES

1. BHASKARAN S. (1964) ESR studies on plant seeds of differential radiosensitivity. I. Effect of water content. *Radiation Botany* (in press).
2. BERGBUSCH V. L. and CALDECOTT R. S. (1963) The effects of preirradiation and postirradiation temperature treatments on the X-ray sensitivity of seeds of *Hordeum*. *Radiation Res.* **20**, 207–220.
3. BERINGER R. and CASTLE J. G. (1950). Magnetic resonance absorption in nitric oxide. *Phys. Rev.* **78**, 581–586.
4. CALDECOTT R. S. (1961) Seedling height, oxygen availability, storage and temperature: Their relation to radiation-induced genetic and seedling injury in barley. In: *Effects of Ionizing Radiations on Seeds*. Intern. At. Energy Agency, Vienna, 1961.
5. EHRENBERG A., EHRENBERG L. and LOEFROTH G. (1962). Radiation induced paramagnetic centers in plant seeds at different oxygen concentrations. *Abhandl. Deut. Akad. Wiss., Berlin. Kl. für Med. Nr.* **1**, 229–231.
6. KÖHNLEIN W. and MÜLLER A. (1960) Ein Doppelhohlraum fuer Präzisionsmessungen der Konzentration freier Radikale. *Z. Naturforsch.* **15b**, 138–139.
7. KÖHNLEIN W. and MÜLLER A. (1962) Absolute yield measurements of radiation produced radicals by electron spin resonance. *Phys. Med. Biol.* **6**, 599–604.
8. NATARAJAN A. T. and AHNSTRÖM G. (1961) Oxygen saturation and dry seed irradiation. *Naturwissenschaften* **48**, 698–699.
9. POWERS E. L., WEBB R. B. and KALETA B. F. (1960) Oxygen and nitric oxide as modifiers of radiation injury in spores of *Bacillus megaterium*. *Proc. Nat. Acad. Sci. U.S.* **46**, 984–993.
10. SPARRMAN B., EHRENBERG L. and EHRENBERG A. (1959) Scavenging of free radicals and radiation protection by nitric oxide in plant seeds. *Acta Chem. Scand.* **13**, 199–200.
11. ZIMMER K. G. (1959) Evidence for free radical production in living cells exposed to ionizing radiation. *Radiation Res.*, Suppl. **1**, 519–529.
12. ZIMMER K. G., KÖHNLEIN W., HOTZ G. and MÜLLER A. (1963) Elektronen-Spin-Resonanzen in bestrahlten Bakteriophagen und deren Bestandteilen I. Mitteilung. *Strahlentherapie* **120**, 161–190.