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ON THE DISTRIBUTION OF RIBOSOME-INACTIVATING PROTEINS AMONGST PLANTS

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ABSTRACT.—The extracts from various parts (mostly seeds) of 56 different plants were examined for inhibition of protein synthesis by a rabbit reticulocyte lysate. Most extracts inhibited protein synthesis with an ID_{50} (concentration giving 50% inhibition) of 100 μg extract protein per ml, or less. The extracts with high activity were partially purified by CM cellulose chromatography. Protein-containing fractions were separated which inhibited protein synthesis and resembled the ribosome-inactivating proteins from plants previously described. Thus, ribosome-inactivating proteins appear to be virtually ubiquitous in plants.

Ribosome-inactivating proteins (RIPs) (1) are proteins, so far identified only in plants, that inactivate eukaryotic ribosomes, acting on them in a less-than-equimolar ratio, i.e., in an apparently catalytic manner. Through a still unknown mechanism, they render the 60S ribosomal subunit incapable of binding the elongation factor 2, thus arresting protein synthesis. In previous papers (2, 3), approximately one-third of the seed-extracts from several plants examined inhibited protein synthesis, at a concentration of seed protein not higher than 100 μg/ml. This inhibitory activity could be accounted for by RIPs which were isolated from the extracts (4-11).

With present experiments, we examined a number of extracts from plant materials, using in some cases a more refined purification procedure. This allowed us to detect the presence of much lower concentrations of RIPs, thus providing better information on the distribution of these proteins amongst plant species. The results showed that most extracts inhibited protein synthesis and that all those examined further contained proteins with properties very similar to those of known purified RIPs, thus supporting the notion that RIPs may be ubiquitous in the plant kingdom (3, 9, 11). It appeared also that a number of RIPs can be purified in large quantities. This may be very useful for the preparation of cytotoxic conjugates of RIPs with antibodies ("immunotoxins") or other suitable carriers, as practiced with gelonin (9, 12, 13) and with the pokeweed antiviral protein (14-17).

EXPERIMENTAL

PLANT MATERIALS.—These were obtained from the sources indicated in Table 1. Seeds were stored at room temperature. Leaves, if not used fresh, were kept at −20°. Latices were diluted, immediately after collection, with 10 volumes of 0.14 M NaCl containing 5 mM sodium phosphate buffer, pH 7.2, and were stored at −20° until use.

CHEMICALS.—Bovine serum albumin was obtained from Sigma Chemical Co., St Louis, MO, and was used as a standard for protein determination. Markers for molecular weight determinations were bovine serum albumin and chymotrypsinogen from Boehringer Mannheim GmbH, Mannheim, West Germany; ovalbumin from Pharmacia Fine Chemicals, Uppsala, Sweden; and reduced modeccin, prepared as described by Stirpe *et al.* (18). All other chemicals were obtained from the same sources as in previous work (3).

PREPARATION OF EXTRACTS.—Seeds, leaves, and roots were homogenized in a blender or with an Ultra-Turrax apparatus with enough phosphate-buffered saline described above (1.5 to 10 ml/g, depending upon the material used) to obtain a reasonably liquid slurry. Extracts were left overnight at 4° on a magnetic stirrer, and on the following morning were strained through cheesecloth and were centrifuged at 28,000 g for 30 min. The supernatant was separated from the sediment and (when present) from a floating layer of fat and is referred to as "crude extract."

CHROMATOGRAPHY.—In all cases but *Asparagus officinalis* seeds, the procedure was as described previously (9). Crude extracts or diluted latices were dialyzed against 40 volumes of 5 mM sodium phos-

phate buffer, pH 6.5, with two changes, for at least 24 h at 4°. Any precipitate formed during dialysis was removed by centrifugation, and the resulting clear supernatant was applied to a CM cellulose (CM 52) column (20 cm × 1.6 cm), previously equilibrated with the same buffer. The column was washed with buffer until the A_{280} of the effluent was below 0.1 and was then eluted with a 0-0.3 M linear NaCl gradient in the same buffer (total volume 500 ml). In the case of *A. officinalis* seeds, a 5 mM sodium phosphate buffer, pH 6.0, was used in an otherwise identical procedure. The A_{280} of the effluent was monitored and 2-ml fractions were collected, and those showing an absorbance were assayed for inhibition of protein synthesis.

PROTEIN SYNTHESIS.—Protein synthesis was determined with a rabbit reticulocyte lysate as described previously (10). Reaction mixtures contained, in a final volume of 125 μl: 10 mM Tris/HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 6 μg of creatine kinase, 0.05 mM amino acids (minus leucine), 0.19 μCi of L-[^{14}C]leucine and 50 μl of a lysate of rabbit reticulocytes. Incubation was at 27° for 5 min. The radioactivity incorporated into protein was determined on 25 μl-samples as described previously (10).

OTHER DETERMINATIONS.—Protein was determined by the method of Lowry *et al.* (19) or spectrophotometrically (20).

SDS-polyacrylamide-gel electrophoresis was performed as described by Laemmli (21) with the following markers for molecular weights: bovine serum albumin (M_r 68,000), ovalbumin (43,000), modeccin B-chain (35,000) and A-chain (28,000), and trypsin inhibitor (21,500).

RESULTS AND DISCUSSION

The effects on protein synthesis of extracts from various parts (mostly seeds) from 56 plant species belonging to 51 genera in 25 families are summarized in Table 1. Of these, 30 crude extracts inhibited protein synthesis with an ID_{50} (concentration giving 50% inhibition) below 100 μg extract protein/ml, 14 had an ID_{50} above 100 μg/ml, and 6 appeared inactive. Nineteen extracts, selected mainly on the basis of high activity and availability of material, were chromatographed on CM 52. In all cases, including two extracts with low activity (*Spinacia oleracea* and *Setaria italica* seeds), at least one, but often more than one, active protein was separated and appeared as one (or more) discrete peak(s) (Figure 1). The inhibitory activity was destroyed by boiling the extracts for 20 min.

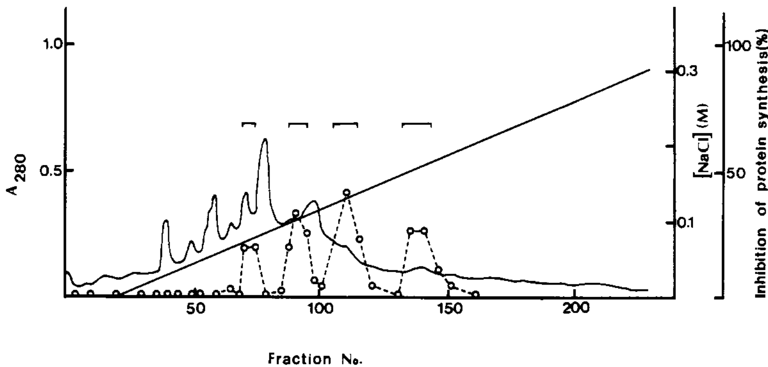

FIGURE 1. Representative chromatography of an extract. The dialyzed leaf-extract of *Beta vulgaris* was chromatographed on a column of CM-cellulose as described in the text. Fractions (2 ml) were eluted with a 0.03 M NaCl gradient (thin line) at 40 ml/hr and their A_{280} was recorded (thick line). The inhibitory activity on protein synthesis (o- - -o) was assayed in the lysate system as described in the text with 10 μl of a suitable dilution of the fractions. Active fractions were pooled as indicated by the horizontal bars.

On SDS-polyacrylamide-gel electrophoresis, a protein band with mobility corresponding to M_r 30,000, approximately, was present in all active peaks, in some cases together with other proteins (Figure 2). When peaks were electrophoretically homo-

TABLE 1. Inhibitory Activity of Extracts on Protein Synthesis by a Rabbit Reticulocyte Lysate

Family and species	Part used (source)[a]	Preparation	Protein (mg/100 g starting material)	ID_{50}[b] (μg protein/ml)	Units (ID_{50}/mg protein)
Amaranthaceae					
Amaranthus caudatus rubens	seeds (1)	crude extract	7,684	1.6	625
Amaryllidaceae					
Agave americana	leaves (2)	crude extract	1,636	2.6	380
		CM 52 0.110 M[c]	0.53	0.1	9,400
Araceae					
Arum italicum	spadix (3)	crude extract	1,965	126	7.9
Araliaceae					
Hedera helix	leaves (2)	crude extract	1,173	5	200
		CM 52 0.083 M	0.13	0.096	10,400
Caryophyllaceae					
Agrostemma githago	seeds (4)	crude extract	7,764	0.63	1,587
		CM 52 0.066 M	8.4	0.018	55,555
		CM 52 0.155 M	34.2	0.014	71,429
		CM 52 0.173 M	18.4	0.017	58,824
Dianthus caryophyllus	seeds (5)	crude extract	7,686	4	250
Lychnis vulgaris	leaves (3)	crude extract	1,156	2.2	454
Saponaria officinalis	seeds (2,3)	crude extract	5,668	0.008	113,636
		CM 52 0.122 M	27.6	0.0017	591,715
		CM 52 0.136 M	62.8	0.0012	806,452
		CM 52 0.156 M	414	0.0011	884,955
		CM 52 0.174 M	10.8	0.004	245,700
		CM 52 0.185 M	38.4	0.007	164,474
		CM 52 0.200 M	115.2	0.0011	869,565
	leaves (2)	crude extract	2,844	0.35	2,857
Saponaria sicula	seeds (6)	crude extract	3,385	0.027	37,037
Silene alba	seeds (7)	crude extract	2,380	0.1	10,000
Chenopodiaceae					
Beta vulgaris	leaves (8)	crude extract	2,445	11	91
		CM 52 0.085 M	2	0.44	2,273
		CM 52 0.110 M	3	0.75	1,333
		CM 52 0.140 M	1.7	0.18	5,555
		CM 52 0.180 M	0.52	0.23	4,348
Chenopodium album	seeds (9)	crude extract	2,540	4.7	213
		CM 52 0.075 M	24	0.1	10,000
Chenopodium amaranticolor	leaves (9)	crude extract	754	8.2	122
		CM 52 0.180 M	3.7	1.05	950

Spinacia oleracea	leaves (8)	crude extract	1,374	142	7
		CM 52 0.110 M	1.7	0.84	1,190
		CM 52 0.200 M	0.25	0.62	1,610
Compositae					
Lactuca vulgaris	leaves (8)	crude extract	346	42.5	23
		CM 52 0.088 M	0.2	0.23	4,348
Matricaria camomilla	seeds (8)	crude extract	3,150	14	71
Cruciferae					
Brassica capitata	leaves (8)	crude extract	517	42.5	23
Cucurbitaceae					
Cucurbita ficifolia	seeds (4)	crude extract	4,215	27	37
Euphorbiaceae					
Croton tiglium	seeds (10)	crude extract	3,600	8	125
		CM 52 0.070 M	2.1	0.03	33,000
Euphorbia characias	latex (3,7)	crude extract	4,250	400	2.5
Hevea brasilensis	seeds (11)	crude extract	3,456	10.8	93
		CM 52 0.130 M	40.3	19	53
		CM 52 0.170 M	5.4	2.3	430
Hura crepitans	latex (3,12)	crude extract	10,125	0.116	8,600
		CM 52 0.095 M	146	0.0034	293,000
Jatropha curcas	seeds (10)	crude extract	4,600	0.35	2,860
		CM 52 0.100 M	208	0.58	1,720
Jatropha glauca	whole fruit (seeds included) (13)	crude extract	208	0.36	2,778
Manihot utilissima	leaves (3)	crude extract	2,160	0.75	1,330
Geraniaceae					
Pelargonium zonale	leaves (2)	crude extract	6,250	440	23
Gramineae					
Setaria italica	seeds (8)	crude extract	549	184	5.4
		CM 52 0.130 M	0.43	6	167
		CM 52 0.150 M	0.62	8	125
		CM 52 0.180 M	0.38	15.2	66
Labiatae					
Lavandula officinalis	leaves (2)	crude extract	3,086	38	26
Salvia officinalis	leaves (2)	crude extract	2,025	28	36
Leguminosae					
Caesalpinia pulcherrima	seeds (14)	crude extract	inactive		
Cicer arietinum	seeds (8)	crude extract	5,400	526	1.9
Robinia pseudoacacia	seeds (1)	crude extract	9,968	80	12.5
		CM 52 0.170 M	2.8	0.44	2,270
		CM 52 0.185 M	2.4	1.68	595
		CM 52 0.255 M	1.12	0.08	12,500
Vicia faba	seeds (8)	crude extract	406	16	62.5

Table 1. Continued

Family and species	Part used (source)[a]	Preparation	Protein (mg/100 g starting material)	ID_{50}[b] (μg protein/ml)	Units (ID_{50}/mg protein)
Liliaceae					
Allium cepa	bracts (8)	crude extract	427	60	16.7
Asparagus aculeatus	seeds (7)	crude extract	4,867	2	500
Asparagus officinalis	seeds (1)	crude extract	1,700	0.29	3,448
		CM 52 0.034 M	23.8	0.013	76,923
		CM 52 0.046 M	7.9	0.011	90,909
		CM 52 0.084 M	17.9	0.005	200,000
Convallaria majalis	leaves (2,3)	crude extract	3,037	4	250
	rhizomes (2)	crude extract	1,110	0.038	26,316
		CM 52 0.054 M	5.6	0.0025	400,000
		CM 52 0.066 M	4.8	0.0035	287,714
	roots (2,3)	crude extract	1,097	1.59	629
Ruscus aculeatus	seeds (7)	crude extract	inactive		
Tulipa sp.	bulbs (1)	crude extract	1,877	71	14
Veratrum niger	leaves (3)	crude extract	1,700	160	6.25
	rhizomes (3)	crude extract	2,109	52	19
Veratrum viridis	leaves (3)	crude extract	2,471	60	17
	rhizomes (3)	crude extract	2,327	400	2.5
Magnoliaceae					
Magnolia japonica	leaves (2)	crude extract	2,605	175	5.71
Moraceae					
Ficus carica	latex (2)	crude extract	4,680	20	50
		CM 52 0.148 M	85.2	46.5	21
		CM 52 0.152 M	58.3	16	62
		CM 52 0.190 M	101	76	13
Maclura pomifera	seeds (2)	crude extract	9,427	50	20
		CM 52 0.040 M	4.1	10	100
Papaveraceae					
Chelidonium maius	seeds (4)	crude extract	1,962	124	8.1
Passifloraceae					
Passiflora subpeltata	seeds (4)	crude extract	3,024	150	6.7
Pinaceae					
Pinus pinea	endosperm (2)	crude extract	3,816	310	3.2
Polypodiaceae					
Drypterix filix	leaves (2)	crude extract	inactive		

Rosaceae					
Fragaria vesca	leaves (2,7)	crude extract	inactive		
Nespilus germanica	seeds (8)	crude extract	inactive		
Prunus communis	fruits (8)	crude extract	inactive		
Rosa sp.	leaves (2)	crude extract	12,720	810	1.2
Rutaceae					
Citrus nobilis	seeds (8)	crude extract	2,193	54.4	18
		CM 52 0.110 M	3.4	1.4	694
Solanaceae					
Solanum lycopersicon	seeds (1)	crude extract	882	32	31
Umbelliferae					
Apium graveolens	stems + leaves (8)	crude extract	317	20	50
Daucus carota	roots (8)	crude extract	234	158	6

[a]The sources of materials were: (1) Ansaloni, San Lazzaro, Bologna; (2) private gardens; (3) The Botanical Garden, University of Bologna; (4) University of London Botanical Supply Unit, Egham, Surrey, UK; (5) Clause Ltd, Chervil, Reading, Berks., U.K.; (6) The Botanical Garden, University of Palermo, Italy; (7) fields; (8) local market; (9) Istituto di Patologia vegetale, University of Bologna; (10) Mr. F.G. Celo, Zweibruecken, West Germany; (11) Malaysian rubber producer's Research Association, Brickendonbury, Hertford, UK; (12) Dr. L.E. Newton, Kumasi, Ghana; (13) University of Karthoum, Sudan; (14) Versepuy, Le Puy, France.

[b]ID_{50}: concentration giving 50% inhibition (estimated by the linear regression analysis).

[c]CM 52 followed by a molarity indicates a peak eluted from a CM cellulose column at that molarity of the NaCl gradient.

geneous and highly concentrated, active proteins were purified and characterized further (22).

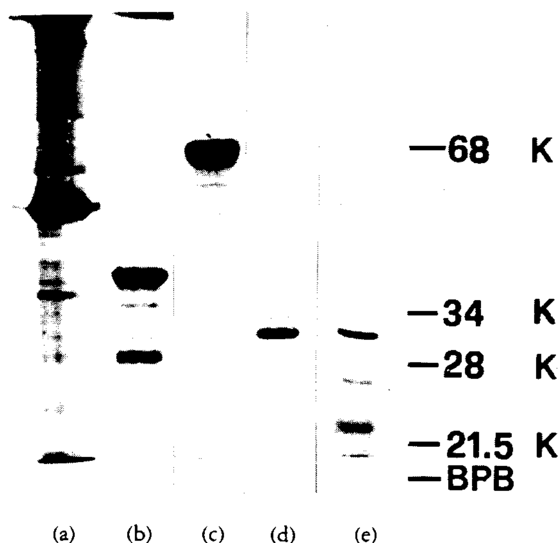

FIGURE 2. Sodium dodecyl sulfate/polyacrylamide/gel electrophoresis. Electrophoresis was performed as described in the text. Migration was from top to bottom, and the bromophenol blue front is indicated BPB. *Beta vulgaris*: (a) crude extract; (b) peak eluted at 0.085 M NaCl; (c) peak at 0.11 M NaCl; (d) peak at 0.14 M NaCl; (e) peak at 0.18 M NaCl.

These results demonstrate that extracts from most plants, including edible ones, inhibit protein synthesis by a lysate of rabbit reticulocytes. In all cases examined further, the inhibitory activity was due to proteins which for several properties (size, chromatographic behavior, inhibitory effect on protein synthesis) resembled the RIPs identified previously (1). Indeed, those proteins which were characterized further appeared to have all the properties of RIPs (22). The presence, in several cases, of more than one active protein is consistent with previous observations (6, 10) and may be due to the existence of isoforms of RIPs.

RIPs appear to be present in many parts (seeds, leaves, roots, latices) of almost all plants examined. In some cases they, although present, may have not been detected because the assay system was not sensitive enough, as it was suggested by Coleman and Roberts (11). Their concentrations vary from one plant to another and among different tissues of the same plant. Seed-extracts were usually more active than extracts from other parts of the same plant, although *Convallaria majalis* was an exception.

Present and previous investigations (2, 3, 11) are too limited to allow generalized conclusions on the distribution of RIPs amongst families and genera, although it seems that seeds from Caryophyllaceae, Cucurbitaceae, and Euphorbiaceae often contain high concentrations of RIPs. The fact that RIPs are virtually ubiquitous and often abundant in plants strongly suggests that they may have an important function. The hypotheses have been proposed that they may act as a defense against parasites or as regulators of protein synthesis (11). All RIPs examined so far have antiviral activity in plants (1). It was suggested that the antiviral action of the pokeweed antiviral proteins is due to damage of ribosomes in virus-infected cells, an effect exerted only on ribosomes from other

plant species (23). This is consistent with the fact that RIPs exert an antiviral effect on heterologous plants only and rules out the possibility that protection against viral infections can be a significant natural function of these proteins. The same inhibition of protein synthesis was exerted by extracts from healthy and virus-infected leaves of *Chenopodium amaranticolor*, thus indicating that RIPs are not induced by viral infections, by difference with the "interferon-like" antiviral factors found in virus-infected plants (24).

RIPs also inhibit the multiplication of animal viruses (8, 25-27). Since these proteins resist proteolytic digestion (9, 22), the hypothesis was formulated that RIPs contained in vegetables eaten raw may contribute to the control of viral infections in the intestine of animals and man (22).

The pokeweed antiviral protein is toxic to tobacco (28) and carrot (29) cells in culture. This, and the fact that RIPs are present in saps, sometimes in high concentration (*Hura crepitans*, 22), led to the suggestion that they may be amongst the factors preventing the establishment of heterografts (29), if grafts were attempted on stocks, the sap of which contained RIPs toxic to the cells of the scions. It is noteworthy that the reasons for the failure of plant heterografts are unknown, although the possibility that stocks could produce substances toxic to the scion was suggested (30). Should these observations be extended and substantiated further, an important role of RIPs may emerge in preventing the success of spontaneous grafts amongst plants, thus preserving the integrity of plant species.

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LITERATURE CITED

1. L. Barbieri and F. Stirpe, *Cancer Surveys*, **1**, 489 (1982).
2. A. Gasperi-Campani, L. Barbieri, E. Lorenzoni, and F. Stirpe, *FEBS Lett.*, **76**, 173 (1977).
3. A. Gasperi-Campani, L. Barbieri, P. Morelli, and F. Stirpe, *Biochem. J.*, **186**, 439 (1980).
4. J.D. Irvin, *Arch. Biochem. Biophys.*, **169**, 522 (1975).
5. W.R. Roberts and T.S. Stewart, *Biochemistry*, **18**, 2615 (1979).
6. L. Barbieri, M. Zamboni, L. Montanaro, S. Sperti, and F. Stirpe, *Biochem. J.*, **186**, 443 (1980).
7. J.D. Irvin, T. Kelly, and J.D. Robertus, *Arch. Biochem. Biophys.*, **200**, 418 (1980).
8. L. Barbieri, G.M. Aron, J.D. Irvin, and F. Stirpe, *Biochem. J.*, **203**, 55 (1982).
9. F. Stirpe, S. Olsnes, and A. Pihl, *J. Biol. Chem.*, **255**, 6947 (1980).
10. F. Stirpe, D.G. Williams, L.J. Onyon, R.F. Legg, and W.A. Stevens, *Biochem. J.*, **195**, 399 (1981).
11. W.H. Coleman and W.R. Roberts, *Biochim. Biophys. Acta*, **696**, 239 (1982).
12. P.E. Thorpe, A.N.F. Brown, W.C.J. Ross, A.J. Cumber, S.I. Detre, D.C. Edwards, A.J.S. Davies, and F. Stirpe, *Eur. J. Biochem.*, **116**, 447 (1981).
13. M. Colombatti, M. Nabholz, O. Gros, and C. Bron, *J. Immunol.*, **131**, 3091 (1983).
14. Y. Mashuo, K. Kishida, and T. Hara, *Biochem. Biophys. Res. Commun.*, **105**, 462 (1982).
15. J.P. Allison, B.W. McIntyre, D. Bloch, G.B. Kitto, and J.D. Irvin, *Fed. Proc.*, **41**, 309 (1982).
16. S. Ramakrishnan and L.L. Houston, *Cancer Res.*, **44**, 201 (1984).
17. M.J. Bjorn, J. Larrick, M. Piatak, and K.T. Wilson, *Biochim. Biophys. Acta*, **790**, 154 (1984).
18. F. Stirpe, A. Gasperi-Campani, L. Barbieri, E. Lorenzoni, L. Montanaro, S. Sperti, and E. Bonetti, *FEBS Lett.*, **85**, 65 (1978).
19. O.H. Lowry, N.J. Rosebrough, A. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
20. V.F. Kalb and R.W. Bernlohr, *Anal. Biochem.*, **82**, 362 (1977).
21. U.K. Laemmli, *Nature*, **227**, 680 (1970).
22. F. Stirpe, A. Gasperi-Campani, L. Barbieri, A. Falasca, A. Abbondanza, and W.A. Stevens, *Biochem. J.*, **216**, 617 (1983).

23. R.A. Owens, G. Bruening, and R.J. Shepherd, *Virology,* **56**, 390 (1973).
24. I. Sela, *Perspect. Virol.*, **11**, 129 (1980).
25. M.A. Ussery, J.D. Irvin, and B. Hardesty, *Ann. N. Y. Acad. Sci.*, **284**, 431 (1977).
26. G.M. Aron and J.D. Irvin, *Antimicrob. Agents Chemother.*, **17**, 1032 (1980).
27. L. Foà-Tomasi, G. Campadelli-Fiume, L. Barbieri, and F. Stirpe, *Arch. Virol.*, **71**, 323 (1982).
28. S. Grasso, P. Jones, and R.F. White, *Phytopat. Z.*, **98**, 53 (1980).
29. M.G. Battelli, E. Lorenzoni, F. Stirpe, R. Cella, and B. Parisi, *J. Exp. Bot.*, **35**, 882 (1984).
30. R. Moore and D.B. Walker, *Am. J. Bot.*, **68**, 831 (1981).

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