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DETECTION OF β-TUBULIN IN TOMATO SEEDS: OPTIMIZATION OF EXTRACTION AND IMMUNODETECTION

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; seed; imbibition; germination; β-tubulin.

Abstract—SDS–PAGE profiles of SDS-denaturated total protein (MODIL) extracts of imbibing tomato seeds showed no large qualitative differences from those of water-soluble (HEPES) proteins. However, the protein concentrations of the HEPES samples were significantly lower than the MODIL ones. The presence of SDS and DTT in MODIL buffer at an alkaline pH were essential for high yields of β-tubulin. The absence of DTT in HEPES extraction buffer may have allowed oxidation and subsequent loss of β-tubulin extracted from root tips of germinated seeds. Optimization of the β-tubulin extraction procedure and improvements of the immunodetection system enabled us to demonstrate the β-tubulin accumulation pattern in whole tomato seeds and in the different tissues. β-Tubulin accumulation in seeds was shown to be tissue-dependent, the highest concentration being found in embryo root tip tissue

INTRODUCTION

Tubulin polypeptides are components of both animal and plant microtubules. Monomers have a M_r of *ca* 55 k each, and form a heterodimer by means of non-covalent, hydrophobic interactions [1, 2]. During the cell cycle and mitosis in higher plant cells, different cell-cycle-stage-dependent microtubule arrays are involved in a wide range of activities including chromosome segregation, cell plate and cell wall formation.

Existing knowledge on tubulins in cells concerns neurotubulins. Evidence of the conserved nature of the peptide between the Eukaryotes is provided is provided by cross-reactivity between neurotubulin antibodies and plant microtubules of *Leucojum* endosperm cells [3], successful higher plant tubulin polymerization *in vitro* [4] and the cross-reactivity of higher plant antibodies with intracellular microtubules in animals cells [5]. Since these discoveries, immunocytochemical, in particular immunofluorescence techniques, have yielded numerous new findings on microtubules and tubulin properties and activities in plants [6–12]. However, procedures have still to be developed and improved to allow the detection of the low tubulin content in plant tissue cells and to overcome its instability during extraction due to endogenous proteases [5]. The improvement of extraction methods, together with Western blot analysis of tubulins fractioned by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis, have been important steps. An effective extraction and detection method of low plant tubulin content has been described using an autoradiographical immunoblotting method [13]. Several other extraction and immunodetection procedures for identification and/or quantification of higher plant tubulins are known, most of them from *in vitro* plant cell cultures and seedlings [10–17].

However, little is known about tubulin from seed tissues. Biochemical and immunological properties of tubulin from monocotyledon endosperm cells have been reported [5], and recently we reported on the pattern of β-tubulin accumulation in relation to cell-cycle activities in imbibing tomato (*Lycopersicon esculentum*) seeds [18]. Here we discuss the extraction of β-tubulin from dry and imbibing tomato seeds, in combination with an improved immunochemiluminescence detection protocol which allowed us to detect β-tubulin levels below 10 ng.

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RESULTS

Protein extraction

Two different protein extraction methods were compared: SDS-denatured total proteins using a modified Laemmli [19] buffer, referred to as MODIL, and water-soluble proteins using HEPES buffer. Significantly lower protein levels were obtained at all four imbibition periods when using HEPES extraction buffer (Table 1). Gel electrophoresis showed that there were no significant qualitative differences in polypeptide patterns between both types of extraction, either from whole seed or from root tip tissue. The only significant difference was observed in the polypeptides patterns of germinated root tip samples compared with all other samples, in that the root tip extracts contained only traces of reserve proteins (results not shown).

Table 1. Amounts of protein extracted from imbibing (0, 24 or 48 hr) seeds using SDS-denaturated total protein (MODIL) or water-soluble protein (HEPES) extraction buffers. Proteins were extracted from whole seed or root tip tissues of dry and imbibing seeds. Data represent the means of two replicates per imbibition period of samples collected from each tissue, i.e. 5 whole seeds or 20 to 30 isolated root tips

Imbibition period	Protein content ($\mu g\ \mu l^{-1}$)	
(hr)	MODIL	HEPES
0	12.3±0.6	2.8±0.8
24	10.1±0.7	3.5±0.5
48	10.2±1.4	3.7±1.3
48[g]	9.1±2.2	2.1±0.6

[g] Seeds germinated after 48 hr of imbibition.

Immunodetection of β-tubulin

Whole seed and root tip protein extracts prepared with MODIL and HEPES buffers were used for immunodetection of β-tubulin at four different seed imbibition times. In all types of protein samples, the monoclonal anti-β-tubulin antibody recognized a protein band with *ca* 55 k M_r, although not at all imbibition periods. In general, the signal was stronger in MODIL samples compared with HEPES samples, and in root tip samples compared with whole seed samples. In root tip samples the signal was detectable starting from 24 hr of imbibition and its intensity increased with the imbibition period, being higher after 48 hr of imbibition in germinated root tip samples, whereas specifically in the corresponding HEPES sample the intensity of the signal was greatly decreased. This was thought to be a result of protease activity which was tested by adding a mixture of protease inhibitors to the HEPES buffer. However, the decrease in the intensity appeared to be due to the buffer composition itself. Later it was proved to be related to the absence of DTT in the HEPES buffer as tested by adding DTT to the buffer. This problem was specific only to root tip samples prepared from germinated seeds (results not shown).

Effects of pH on β-tubulin extraction

To study the effect of pH on the extraction efficiency of β-tubulin from seed tissues, root tip protein extracts were prepared with MODIL buffer, not only at pH 6.8, but also at pH 7.5 and 9.0. A blot containing extracts of three different seed imbibition periods was immunodetected for β-tubulin (Fig. 1). At pH 6.8, β-tubulin was detectable only in samples of 24 hr or longer imbibition periods (Fig. 1, lanes 5–7). However, at pH 7.5, a weak β-tubulin signal was also

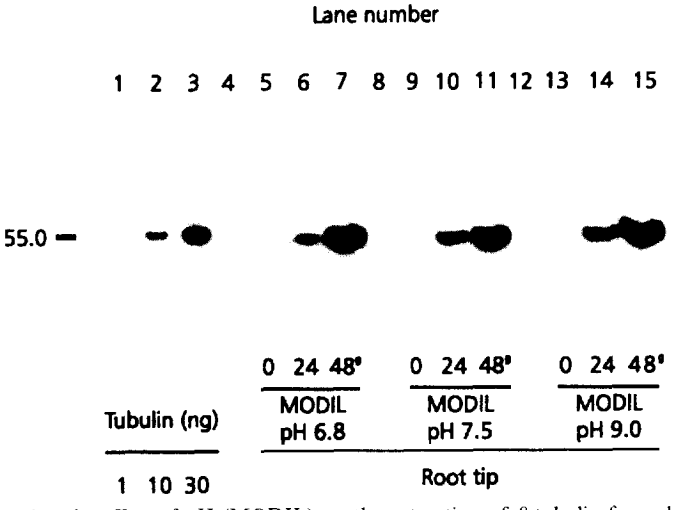

Fig. 1. Immunoblot showing the effect of pH (MODIL) on the extraction of β-tubulin from dry and imbibing (0, 24 or 48 hr) tomato seeds. The gel was loaded with 20 μg of proteins extracted from root tip tissue. Lanes 1–3, pure tubulin; lanes 5–7, MODIL pH 6.8; lanes 9–11, MODIL pH 7.5; lanes 13–15, MODIL pH 9.0. [g]Seeds germinated after 48 hr of imbibition.

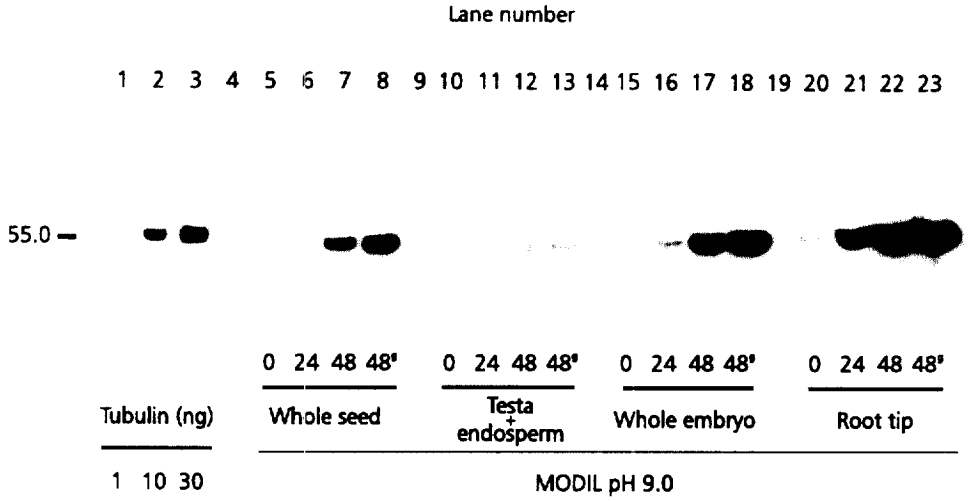

Fig. 2. Immunoblot showing the β-tubulin accumulation pattern (MODIL, pH 9.0) in different tissues of dry and imbibing (0, 24 or 48 hr) tomato seeds. The gel was loaded with 20 μg of proteins extracted from whole seed, testa + endosperm, whole embryo or root tip tissues. Lanes 1–3, pure tubulin; lanes 5–8, whole seed; lanes 10–13, testa + endosperm; lanes 15–18, whole embryo; lanes 20–23, root tip. [a]Seeds germinated after 48 hr of imbibition.

detectable in the dry, non-imbibed sample (Fig. 1, lanes 9–11). At pH 9.0, the signal was even stronger in all samples (Fig. 1, lanes 13–15).

β-Tubulin accumulation pattern in different seed tissues

A blot containing whole seed, testa + endosperm, whole embryo, and root tip protein extracts prepared with MODIL buffer at pH 9.0 was immunodetected for β-tubulin to obtain its accumulation pattern in these different tomato seed tissues during imbibition (Fig. 2). Compared with samples prepared with MODIL buffer at pH 6.8 (results not shown), β-tubulin could now be detected in whole seed samples, but still only in 48 hr imbibed and germinated samples (Fig. 2, lanes 7 and 8). Only very low and about equal levels could be detected in the testa + endosperm from 48 hr imbibed and germinated seed samples (Fig. 2, lanes 12 and 13). In whole embryo samples, β-tubulin was detected after 24 hr imbibition. Its signal increased after 48 hr of imbibition and was highest in embryos from seeds which had germinated after 48 hr imbibition (Fig. 2, lanes 16–18). A similar pattern, but beginning with the dry, non-imbibed sample was detected in isolated root tips (Fig. 2, lanes 20–23).

Effect of protein content loaded

To check the possibility of obtaining a β-tubulin accumulation pattern similar to the one of root tip samples from the latter experiment, a blot was produced from a gel loaded with 70 μg in 40 μl wells of MODIL pH 9.0 whole seed protein extracts, instead of 20 μg in 20 μl wells. The immunodetected blot now allowed us to obtain such a pattern, using whole seed extracts (Fig. 3, lanes 5–8).

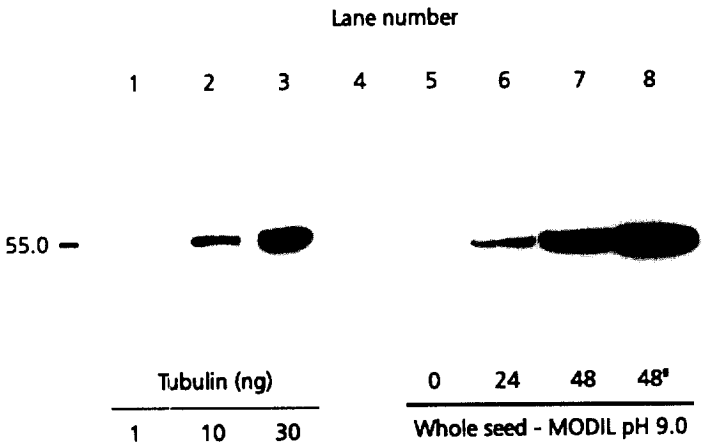

Fig. 3. Immunoblot showing the β-tubulin accumulation pattern in dry and imbibing (0, 24 or 48 hr) tomato seeds. The gel was loaded with 70 μg of proteins extracted from whole seed. Lanes 1–3, pure tubulin; lanes 5–8, MODIL (pH 9.0) whole seed. [a]Seeds germinated after 48 hr of imbibition.

DISCUSSION

It is possible to detect tubulin in *ca* 50 mg of fresh plant material [13]. However, seeds often imply much smaller amounts of material with even smaller tubulin contents.

Although we have recently reported on the immunochemiluminescence detection of β-tubulin in tomato seeds imbibed for 48 hr or more [18], improvement of the protein extraction and immunodetection procedures was required in order to detect lower β-tubulin contents. Since microtubules are present in all stages of a typical plant cell cycle [20], a constitutive amount of β-tubulin was expected to exist in dry seeds and in seeds imbibed for shorter periods.

The loss of the β-tubulin signal in the HEPES germinated root tip extract (not shown) was unexpected, since the signal could be detected in earlier imbibition periods in a pattern similar to that observed for the MODIL samples, which allowed an effective detection of β-tubulin in germinated root tips. Moreover, germination requires cell expansion for which β-tubulin is a key component [18]. Consequently, the loss of β-tubulin in the HEPES germinated root tip extract was considered in artefact which could be due, for instance, to activity of proteases [5] which could be acting at the moment of radicle protrusion and seedling growth. However, we have shown that the problem was due to the lack of DTT in the HEPES buffer.

The cells of the embryonic root tips apparently become increasingly accessible to O_2 once the radicle has protruded through the seed coat [20, 21], therefore becoming highly susceptible to oxidation. Oxidation of β-tubulin sulphhydryl groups into disulphide bridges may have induced its loss during isolation and extraction, since the addition of DTT to the HEPES buffer allowed the recovery of the β-tubulin signal (not shown). Tubulin polypeptides contain a considerable number of sulphhydryl groups [22–26]. Sulphhydryl groups were found to be important for *in vivo* polymerization of tubulins and assembly of microtubules, being influenced by intracellular levels of reduced glutathione [27, 28]. Glutathione is a cellular sulphhydryl component essential in reductive processes for synthesis and degradation of proteins [29]. Oxidation of glutathione has been reported in tomato seeds as a result of ageing [30]. Decreasing the number of tubulin free sulphhydryls in human cells inhibited tubulin polymerization, but the process could be immediately reversed by the addition of DTT [31]. DTT is known to prevent oxidation of other proteins as well, by reduction of disulphide bridges [32]. It was also found that DTT should be included in the isolation buffer of tubulin from epicotyls of *Vigna angularis* [15]. When using MODIL buffer without DTT, SDS alone could denature the polypeptides fast enough to promote some degree of β-tubulin solubilization, but it was not as effective as when DTT was present in the buffer (not shown).

It may be concluded that the combination of SDS-denaturing and DTT-reducing effects of the MODIL buffer gave the best solubilizing and preserving effect on β-tubulin extraction from tomato seeds. Nevertheless, the very low content of β-tubulin expected to exist in dry (non-imbibed) seed tissues was only extractable when the pH of the MODIL buffer was increased to the limit of its buffering capacity, pH 9.0 (Fig. 1). Alkaline pHs were shown to be more effective, not only in tubulin gel mobility [5], but also in its extraction when hydrophobically associated with membranes [33].

The modified procedure allowed us to detect less than 10 ng of tubulin in *ca* 5 mg of seed material, i.e. 20 embryonic root tips. The relatively high β-tubulin content detected in the imbibing root tip region (Fig. 2) expressed strong correlation with the observed high cell-cycle activities in this same region [34, 35]. It may be assumed that tubulin from testa + endosperm cells seemed to exist in a stable form not related to cell cycle activation, since β-tubulin showed relatively very little or no expression during seed imbibition. The β-tubulin accumulation pattern observed in root tips could only be reproduced in whole seeds when a much higher total protein content was loaded on the gel (Fig. 3).

Summarizing, we have been able to develop an efficient method for the extraction of β-tubulin from tomato seeds. Its accumulation pattern detected during seed imbibition and germination could be well defined. The method has also been applied successfully to seeds of other species such as neem (*Azadirachta indica* A. Juss) [36], cabbage (*Brassica oleracea* L.) and pea (*Pisum sativum* T.) (unpublished data). Tubulin accumulation in imbibing seeds appears to play an important role in seed germination and may therefore be considered as a potential marker of the progress of germination [18].

EXPERIMENTAL

Seed material and imbibition conditions. Seeds of tomato (*Lycopersicon esculentum* M., cv Moneymaker), batch 1992, were dried over a satd $CaCl_2$ soln for 2 days at 20° and 32% relative humidity (seed moisture content 6.3±0.1%, fr. wt basis) [37] and stored in a moisture-proof container at 5° until use. Seed imbibition took place with seeds placed on top of filter paper soaked with H_2O in a sealed Petri dish. Seeds were then kept in an incubator in continuous darkness and 25° from 0 hr (dry seeds) up to 48 hr, when seeds started to germinate, presenting visible radicle protrusion with a maximum of *ca* 1 mm.

Protein extraction methods. After seeds were allowed to imbibe for the appropriate time, samples consisting of 5 whole seeds, 5 testas + endosperms, 5 whole embryos, or 20 to 30 root tips excised from the seeds, were used for protein extraction. The different tissues were put in separate ice-cold Eppendorf reaction assay tubes. After *ca* 15 min, i.e. the average time required to isolate the tissue, the tubes were placed

in liquid N_2 and subsequently the seed material was ground to a powder. Two protein extraction methods were used to prepare the protein samples, i.e. (a) SDS-denaturated total protein extraction and (b) H_2O-soluble protein extraction. In the former method, we added 160 μl (for whole seed, testa + endosperm and whole embryo) or 40 μl (for root tips) of MODIL buffer (62.5 mM Tris–HCl, 2% SDS, 15 mg ml^{-1} DTT and 7% glycerol, pH 6.8–9.0), directly to the frozen powder which was further processed as described in ref. [18]. In the latter method, in the first step we added 80 μl for whole seeds or 20 μl for root tips of Hepes buffer (100 mM Hepes, pH 7.0) directly to the frozen powder. After mixing, the samples were incubated for 10 min at room temp. and centrifuged for 7 min at 17000 *g*. In the second step, the supernatants were mixed with an equal amount of ×2 concd MODIL buffer, and boiled for 10 min. This method we refer to as the HEPES buffer extraction method.

Protein quantitation. Protein concns of all supernatants were measured following micro-protein assay procedures (Bio-Rad), modified from the methods described in refs [38, 39], using BSA as standard.

Electrophoresis and Electroblotting. Electrophoresis was done in one-dimensional polyacrylamide gels with 20 or 70 μg of proteins samples loaded and sepd on a precast 8–18% SDS gradient ExcelGel, according to the manufacturer (Pharmacia). Three different amounts of pure bovine brain tubulin, 1, 10 and 30 ng, were also loaded as reference samples. After PAGE, proteins were electroblotted for immunodetection of β-tubulin, but with the transfer being done in only 2 hr, instead of overnight [18].

Chemiluminescence immunodetection of β-tubulin. Immunodetection of β-tubulin was done as described in ref. [18], with some modifications. The incubation period of the immunoblots in blocking soln, as well as the incubation in the mouse monoclonal anti-β-tubulin antibody soln, was increased to 2 hr. All washing steps were done with large vols of washing soln (*ca* minimum of 100 ml for a 100 cm^2 membrane) to efficiently avoid unspecific binding of the secondary antibody and background on the blots. Films were exposed for different periods of time, varying from 30 sec to a maximum of 8 min. All other immunodetection steps were kept the same.

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REFERENCES

1. Sackett, D. L. and Lippoldt, R. E., *Biochemistry*, 1991, **30**, 3511.
2. Fosket, D. E., Tonoike, H., Han, I. S. and Colón, A., in *Morphogenesis in Plants: Molecular Approaches*, ed. K. A. Roubelakis-Angelakis and K. Tran Thanh Van. Plenum Press, New York, 1993, p. 55.
3. Franke, W. W., Seib, E., Osborn, M., Weber, K., Herth. W. and Falk, H., *Cytobiologie*, 1977, **15**, 24.
4. Morejohn, L. C. and Fosket, D. E., *Nature*, 1982, **297**, 426.
5. Piquot, P. and Lambert, A. M., *Plant Physiology*, 1988, **132**, 561.
6. Wick, S. M., Seagull, R. W., Osborn, M., Weber, K. and Gunning, B. E. S., *Journal of Cell Biology*, 1981, **89**, 685.
7. Clayton, L. C. and Lloyd, C. W., *European Journal of Cell Biology*, 1994, **34**, 248.
8. Young, T., Hymans, J. S. and Lloyd, C. W., *Plant Journal*, 1994, **5**, 279.
9. Binarova, P., Cihalikova, J. and Dolosel, J., *Cell Biology International*, 1993, **17**, 847.
10. Liu, B., Marc, J., Joshi, H. C. and Palevitz, B. A., *Journal of Cell Science*, 1993, **104**, 1217.
11. Liu, B., Joshi, H. C., Wilson, T. J., Silflow, C. D., Palevitz, B. A. and Snustad, D. P., *Plant Cell*, 1994, **6**, 303.
12. Schmit, A. C., Soppin, V., Chevrier, V., Job, D. and Lambert, A. M., *Chromosoma*, 1994, **103**, 343.
13. Fukuda, H. and Iwata, N., *Plant Cell Physiology*, 1986, **27**, 273.
14. Morejohn, L. C. and Fosket, D. E., *Nature*, 1984, **297**, 426.
15. Mizuno, K., Koyama, M. and Shibaoka, H., *Journal of Biochemistry*, 1981, **89**, 329.
16. Mizuno, K., *Cell Biology Interactions Reports*, 1985, **9**, 13.
17. Laporte, K., Rossignol, M. and Trass, J. A., *Planta*, 1993, **191**, 413.
18. Castro, R. D. de, Zheng, X., Bergervoet, J. H. W., Vos, C. H. R. de and Bino, R. J., *Plant Physiology*, 1995, **109**, 499.
19. Laemmli, U. K., *Nature*, 1970, **227**, 680.
20. Goddard, R. H., Wick, S. M., Silflow, C. D. and Snustad, D. P., *Plant Physiology*, 1994, **104**, 1.
21. Callis, J., *Plant Cell*, 1995, **7**, 845.
22. Renaud, F. L., Rowe, A. J. and Gibbons, J. R., *Journal of Cell Biology*, 1968, **36**, 79.
23. Stephens, R. E., *Journal of Molecular Biology*, 1970, **47**, 353.
24. Lee, J. C., Frigon, R. P. and Timasheff, S. N., *Journal of Biological Chemistry*, 1973, **248**, 7253.
25. Eipper, B., *Journal of Biological Chemistry*, 1974, **249**, 1407.
26. Mellon, M. G. and Rebhuhn, L. I., *Journal of Cell Biology*, 1976, **70**, 226.
27. Oliver, J. M., Albertini, D. F. and Berlin, R. D., *Journal of Cell Biology*, 1976, **71**, 921.
28. Liebmann, J. E., Hahn, S. M., Cook, J. A., Lipschultz, C., Mitchel, J. B. and Kaufman, D. C., *Cancer Research*, 1993, **53**, 2066.

29. Meister, A. and Anderson, M. E., *Annual Review of Biochemistry*, 1983, **52**, 711.
30. De Vos, C. H. R., Kraak, H. L. and Bino, J. R., *Physiologia Plantarum*, 1994, **92**, 131.
31. Ikeda, Y. and Steiner, M., *Biochemistry*, 1978, **17**, 3454.
32. Sepaio, M. P. and Meunier, J. C. F., *Journal of Agriculture and Food Chemistry*, 1995, **43**, 568.
33. Beltramo, D. M., Nunez, M., Alonso, A. D. and Barra, H. S., *Molecular and Cell Biochemistry*, 1994, **141**, 57.
34. Bino, R. J., De Vries, J. N., Kraak, H. L. and Van Pijlen, J. G., *Annals of Botany*, 1992, **69**, 231.
35. Bino, R. J., Lanteri, S., Verhoeven, H. A. and Kraak, H. L., *Annals of Botany*, 1993, **72**, 181.
36. Sacandé, M., Groot, S. P. C., Hoekstra, F. A., De Castro, R. D. and Bino, R. J., *Seed Science Research*, 1997, **7**, 161.
37. International Seed Testing Association, *Seed Science Technology*, 1993, **21**, 25.
38. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *Journal of Biological Chemistry*, 1951, **193**, 265.
39. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.