African Journal of Biotechnology Vol. 7 (10), pp. 1468-1472, 16 May, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB07.488
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Photoinhibition influences protein utilisation during seed germination in *Cleome gynandra* L.

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Accepted 30 August, 2007

Seed storage proteins are mobilised during germination, especially at radicle protrusion. The objective of this study was to examine changes in protein expression during germination of *Cleome gynandra* in the presence or absence of light and at constant or alternating temperatures. Seeds were germinated at alternating 30/20 or 20°C in darkness and in continuous white light. Four prominent proteins of 28 - 32 kDa and 18 - 20 kDa, and a fifth low molecular weight protein of 13 kDa were observed in seeds harvested at brown pod maturity. These proteins remained unchanged during photoinhibition as shown on SDS-PAGE gels. Photoinhibition is a pseudodormancy condition during which seed storage proteins are not utilised.

Key words: *Cleome gynandra,* photoinhibition, seed germination, seed storage proteins.

INTRODUCTION

Seed dormancy may be imposed by prevailing environmental conditions, especially during seed maturation. Seeds that germinate normally in the dark but their germination is inhibited by light are referred to as negatively photoblastic (Gutterman et al., 1992; Bewley and Black, 1994; Baskin and Baskin, 1998). It was observed that the inhibitory effect of light in *Nemophila* is probably on cell elongation (Bewley and Black, 1994), which would mean that the expression of some proteins that facilitate or enhance this process is affected. *Cleome gynandra* exhibited negative photosensitivity on exposure to continuous white light beyond 12 h at 20°C during germination, but photoinhibition was greatly reduced at alternating 20/30°C or at constant 30°C (Ochuodho and Modi, 2005).

Photoinhibition can be compared to thermoinhibition, seed priming and pre-chilling treatments because in these cases the seeds do not germinate. However, Hills et al. (2001) showed that 10 polypeptides, which were expressed during thermoinhibition in *Tagetes minuta*, disappeared when these seeds were transferred to optimal germination temperature. Although there are many reports of increased protein levels during seed priming (McDonald, 1999; Wu et al., 2003), 2-dimension SDS-PAGE indicated that there were no specific priming-induced proteins. Guy and Black (1998) showed that some proteins varied in abundance during imbibition and germination in wheat seeds, but they did not identify the proteins. According to Gallardo et al. (2001) the abundance of 14 polypeptides changed during germination *sensu stricto*, six of which belonged to α- and β-cruciferins. The authors identified polypeptides whose abundance increased during priming as degradation products of 12 S cruciferin β-subunits, which suggested that there was already mobilization of these proteins.

Photoinhibition has also been suggested when excessive light has caused photosynthesis to cease (Gray et al., 2003; Govindachary et al., 2004). This is dependent on environmental conditions and is accelerated at low or high temperatures and during drought, when the D1 protein is degraded followed by the inhibition of the electron transport reactions (Mamedov and Styring, 2003; Bergo et al., 2003). It is unclear whether the phenomenon of photoinhibition in photosynthesis is similar to that in seed germination.

The objective of the present study was to examine the mechanism of photoinhibition during seed germination.

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The response of seeds grown in different temperature conditions to light and temperature interactions during germination was investigated.

MATERIALS AND METHODS

Plant material

Two seed lots of *C. gynandra* were each donated by Kenya Seed Company (KSC), Kenya and Agricultural Research Council (ARC), South Africa in 2002. Fresh seeds were grown in glasshouses at day/night temperatures of 21/16, 27/22 and 33/28°C, and harvested when the pods had turned brown. The glasshouses experienced natural day length during the months of July to October 2002 (Pietermaritzburg: 29°35′S30°25′E) and a controlled relative humidity of 60%.

Seed germination

Four replicates of 50 seeds were germinated at 20°C or alternating 20/30°C (16 h night/8 h day, respectively) in continuous white light or in darkness (Labcon LTGC 20 - 40; Johannesburg, South Africa). Seeds were removed from incubation after 24, 48, 72 or 96 h and non-imbibed seeds were used as control. The seed lots from the glasshouse were incubated at 20°C in continuous light for 3 or 7 or ten days (Photoinhibition) and protein analysis of seeds that had been photoinhibited for 7 d is reported. The seeds for protein analysis were ground in liquid nitrogen and stored at -20°C until extraction was performed. Seeds were considered germinated when radicle protrusion was evident.

Extraction of proteins

Extraction buffer was prepared by dissolving 21.02 g urea and 7.61 g thiourea in distilled water. To this buffer was added 0.2% v/v CHAPS, 1% v/v carrier ampholytes (pH 3 - 10), 18 mM Tris, one tablet proteinase inhibitor (Roche Diagnostics GmbH), 53 U/ml DNase and 4.9 U/ml RNase and lastly 1% w/v dithiothreitol (DTT) (Gallardo et al., 2001; Görge et al., 2003). Fifty seed were ground and 1.2 ml of the extraction buffer was added in a 1.5 ml centrifuge tube. Extraction was carried out at room temperature for 1 h with intermittent shaking. The extract was centrifuged (Beckman J2-21M) at a speed of 32,000 g for 10 min at 4°C to obtain a clear supernatant. Protein content of the extracts was determined using the Pierce Micro BCA™ Protein Assay Reagent Kit with bovine serum albumin (BSA) as a standard, according to the manufacturer's instructions.

Polyacrylamide gel electrophoresis

To separate the proteins on weight basis, the SDS-PAGE gel (Laemmli, 1970) system was employed, according to the manufacturer's instructions (Amersham Pharmacia Biotech SE 260). The proteins were resolved with 10% SDS-PAGE gels cast with a 7 cm resolving gel and 1 cm stacking gel. Samples of 20 µl protein extract were loaded and the gels were run at 300 V, 20 mA constant current for 3 h.

Two-dimensional SDS-PAGE was performed according to the manufacture's instructions (Pharmacia Biotech SE 260 System). The first dimension isoelectrofocusing (IEF) was performed on IPG strips (immobilized pH gradient) loaded with protein extracts; each strip with one sample extract. The Immobiline™ DryStrip (pH 3 - 10, 11 cm long) was rehydrated with 100 µl of sample dissolved in 100 µl rehydration solution (8 M Urea, 2% CHAPS, 0.2% Ampholyte, 0.5% IPG buffer and 0.5% DTT) in the reswelling tray overnight at about 22°C. The strip was washed three times in distilled water and placed in the IEF apparatus. The conditions for IEF on Multiphor II System (Pharmacia) were 500V for 4.5 h in Phase I and II and 2000 V for 11.5 h in Phase III and IV, giving a total run time of 16 h, 20100 Vh.

Two-step equilibration procedures followed the isoelectrofocusing of the IPG strip before the second dimension electrophoresis was performed. In the first step the IPG strip was placed into a tube with 20 ml equilibration solution I containing equilibration buffer (6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8) plus 50 mM DTT and shaken for 10 min. The strip was removed and placed into another test tube with 20 ml equilibration solution II containing equilibration buffer plus 4% (w/v) iodoacetamide and bromophenol blue for another 10 min on the shaker. The strip was removed and rinsed in distilled water and placed on moist filter paper to dry. The strip was placed horizontally on top of a 10% SDS-PAGE gel of 1 mm diameter, cast without the stacking gel, at a height of 10 cm. The second dimension was run vertically at 300 V and 60 mA constant current for 2 h. The gels were stained with standard Coommasie (R250) blue overnight, followed by de-staining with Destain II until the gel background became clear. Then the gels were stained with Silver stain with minor modification, where DTT reduction was incorporated to improve reproducibility (Hills et al., 2001). The gels were viewed through VersaDoc Imaging System (Model 4000, Bio-Rad) and the image analysed with the computer package Quantity One.

Statistical analysis

Analysis of variance was used to compare the differences between seed lots ARC, KSC and those obtained from green, yellow and brown pods. Band intensity of gels obtained from seeds whose germination was stopped at various intervals and measured with Quantity One, was also analysed. GenStat statistical package (2000) was used to analyse germination percentages obtained and to compute standard deviations used to obtain the error bars.

RESULTS

Protein bands of KSC seeds incubated at 20/30°C in darkness and in the presence of white light had diminished by 72 h of germination (Figure 1). However, the analysis of band intensity using the computer package AnalySIS showed that the proteins of seeds germinated in darkness diminished faster (24 h) than the bands of seeds incubated in the presence of light (48 h) (Figure 2). Analysis of variance of the bands showed that band intensities at 24 h in darkness were significantly lower than those in light ($P < 0.05$). By 72 h of germination, the intensity of the bands in both light and dark-germinated seeds was less than half the original values (Figure 2).

Cleome seeds incubated at 20°C in continuous light failed to germinate or had low germination. The seeds did not show any protein accumulation change on SDS-PAGE analysis but showed protein bands similar to those

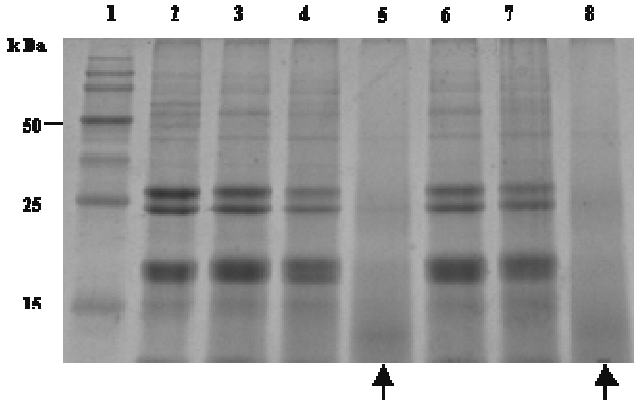

Figure 1. Analysis of the proteins of KSC seeds with 10% SDS-PAGE gel. Molecular weight marker (Lane 1); dry seeds (Lane 2); seeds germinated at alternating 20/30 °C in continuous white light for 24 h (Lane 3), for 48 h (Lane 4) and 72 h (Lane 5); seeds germinated at alternating 20/30 °C in darkness for 24 h (Lane 6), for 48 h (Lane 7) and 72 h (Lane 8). Arrows indicate the lanes where the protein expression diminished.

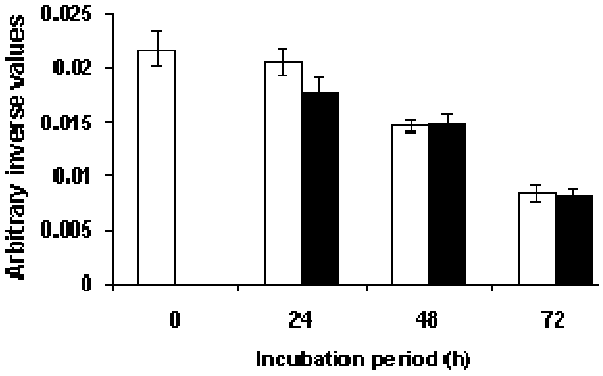

Figure 2. Analysis of band intensity of the gel in Figure 1 using computer soft image system (AnalySIS). Seeds were germinated at alternating 20/30°C with light (□) or in darkness (■). Seed proteins were extracted at intervals and analysed by SDS-PAGE. Error bars represent SD, n =30.

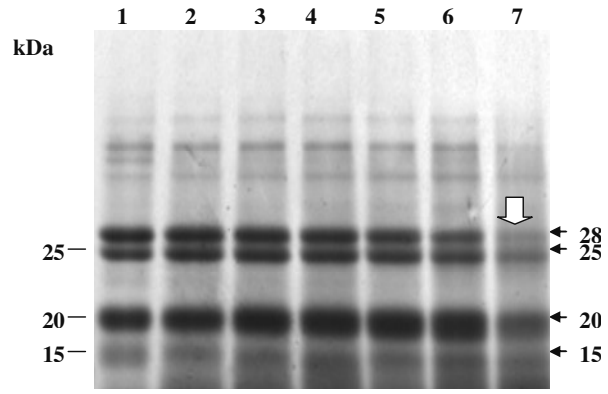

Figure 3. Seeds from KSC germinated at 20°C in continuous light and in darkness. Lane 1, dry seed; Lanes 2, 3 and 4, seeds incubated with light for 48, 72 and 96 h, respectively; Lanes 5, 6 and 7, seeds incubated in darkness for 48, 72 and 96 h, respectively. Open arrow indicates the band that diminished and closed arrows indicate the proteins observed.

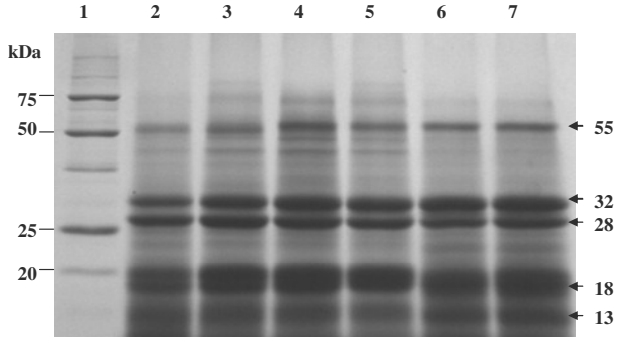

Figure 4. Seeds were grown in glasshouses at day/night temperatures of 21/16, 27/22 and 33/28°C, harvested when the pods were brown and analysed with 10% SDS-PAGE. Lane 1, molecular weight marker. Proteins from dry seeds produced at 21/16, 27/22 and 33/28 °C are shown in Lanes 2, 3 and 4, respectively. Proteins from seeds produced at 21/16, 27/22 and 33/28°C, and incubated at 20°C in continuous white light for 7 d are shown in Lanes 5, 6 and 7, respectively. Arrows indicate the sizes of proteins that were differentially expressed due to germination treatment.

of the dry ungerminated seeds (Figure 3). However, the high molecular weight proteins of 28 kDa began diminishing by the fourth day (96 h) in the seeds that were germinated in darkness.

Photoinhibition of seed germination for up to 7 days neither changed the number nor band intensity of the storage proteins expressed (Figure 4). The protein profile for dry seeds harvested from the glasshouses was similar to that of the seeds incubated at 20°C in continuous light for 7 d, although there were slight indications of diminished content of the heavy bands between 40 - 50 kDa in lanes 6 and 7. The 2-D gels of dry seeds (Figures 5) and photoinhibited seeds (Figure 6) showed the same polypeptides but they differed slightly in content. Due to the reduction activity of DTT, the polypeptides observed in 2-D gels are within the molecular weights 13, 18 - 20 and 28 - 32 kDa only.

DISCUSSION

The seed storage proteins from KSC seed lot diminished earlier during germination in darkness compared to those in continuous white light (Figures 1 and 2). Ochuodho and Modi (2005) showed that seed germination of *C. gynandra* is negatively photosensitive and germinates better and faster in darkness. It has been documented severally that seed storage proteins are mobilised during

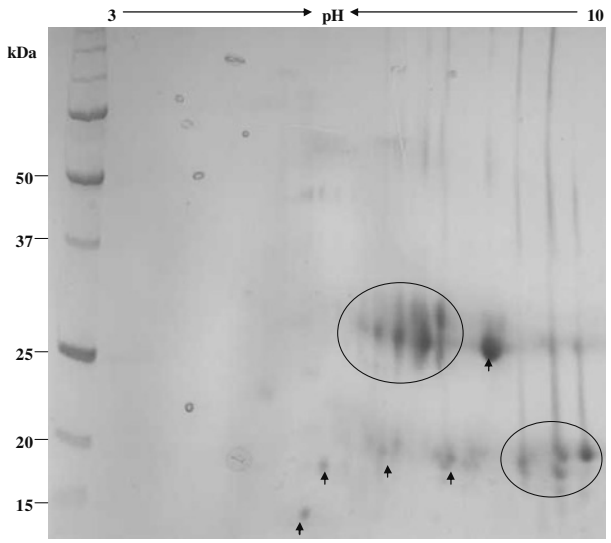

Figure 5. Two-dimensional polyacrylamide gel showing polypeptides isolated from dry seeds of *C. gynandra* produced in glasshouse at day/night temperatures of 27/22°C. Prominent spots are encircled and marked with arrows. These spots correspond with the bands observed in Figure 3.

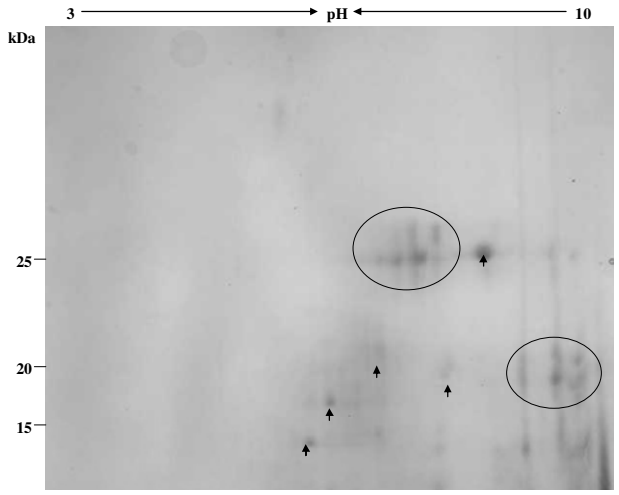

Figure 6. Two-dimensional polyacrylamide gel showing polypeptides isolated from seeds of *C. gynandra* incubated on moist filter papers at 20°C in continuous white light for 7 d. The seeds were produced in a glasshouse at day/night temperatures of 27/22°C. The prominent polypeptides correspond with those shown in Figure 4.

radicle protrusion. Guy and Black (1998) demonstrated that the content of stored and new mRNA in wheat embryos changed depending on seed vigour, while Xia et al. (2002) showed that after breaking dormancy in yellow-cedar, protein bands of the seed lot with higher percent germination diminished faster. Similarly, Klimaszewska et al. (2004) observed that the bands of the prominent protein complex 46-49, 38.2-40, 25-27, 27-29 and 22.5-23.5 kDa disappeared rapidly during germination in the zygotic embryos of *Pinus strobus*.

Seed germination proceeds slowly at low temperatures and the accumulation of proteins in seeds germinated in darkness did not diminish immediately (Figure 3). The expression of the 28 kDa protein gradually decreased as the expression of the 20 kDa protein gradually increased, and the seed progressed towards germination by 96 h. The observation agrees with Gallardo et al. (2001) on the increased accumulation of the subunits of seed storage proteins in *Arabidopsis*. Bewley and Black (1994) observed that the mobilisation of reserve proteins began with the hydrolysis of the larger molecules into smaller subunits. This observation apparently contradicts the findings by Guy and Black (1998) that the number of polypeptides increased during imbibition of wheat in darkness. The authors did not identify the proteins but seemed to be dealing with enzymes and mRNAs involved in the germination process.

A comparison between the protein expressed in dry ungerminated seeds and those from seeds that had failed to germinate at 20°C in continuous white light showed no difference (Figures 4, 5 and 6). During thermoinhibition, Hills et al. (2001) showed that new specific polypeptides were expressed in *Tagetes minuta* seeds and that the proteins disappeared during subsequent germination. Analyses of seed proteins in *C. gynandra* by SDS-PAGE showed consistently five bands of M_r 28-32, 18-20 and 13 kDa, the content of which did not change during photoinhibition, contrary to Russo and Biles (1996) and Hills et al. (2001). The 13 kDa protein was prominent in the seeds from brown pods produced in both the field and the glasshouse conditions. The accumulation of this protein increased after physiological maturity, as it was not observed in immature and physiologically mature seeds (Ochuodho, 2005). It was more abundant in the seed lots from brown pods, which were more sensitive to photoinhibition. The protein was shown to increase during seed maturation and diminish at radicle protrusion (Ochuodho et al., 2006).

In conclusion, these results indicate that during the germination of the seeds of *C. gynandra* seed storage proteins are mobilised. Neither the protein profiles nor the protein content changed during photoinhibition. The content of the four proteins of M_r 28-32 and 18-20 kDa consistently observed in mature seeds of *C. gynandra* did not change during photoinhibition. It can be stated that seed storage proteins are not utilised during photoinhibition in white light.

ACKNOWLEDGEMENTS

The authors are grateful to ARC Roodeplaat, South Africa and Kenya Seeds Company, Kenya for seeds and also NRF, through the University of KwaZulu Natal and MHO-Seed Technology Project, through Moi University, Kenya, for funds.

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