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Quantitative immunochemistry using IgG and IgY against plant hormones

Cleiton Mateus Sousa[1], Flávia Venâncio da Silva[2], Ricardo Motta Miranda[3] and Ronald Bastos Freire*

[1]Goiano Federal Institute (Instituto Federal Goiano – IFGoiano), Ceres Campus, Rod, GO 154, km 03, Zona Rural, 763000-000, Ceres – GO – Brazil.
[2]Department of Sciences, College of Teacher Education – UERJ, Rua Dr. Francisco Portela, 1470 – Paraíso São Gonçalo, RJ CEP 24435-000.
[3]Plant Tissue Culture Laboratory, Department of Plant Sciences, Agronomy Institute, Federal Rural University of Rio de Janeiro (Universidade Federal Rural do Rio de Janeiro – UFRRJ), BR 465 km 07, 23890-000, Seropédica - RJ – Brazil.

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The present study focuses on obtaining and comparing IgY and IgG antibodies against indoleacetic acid (IAA), zeatin (Z) and isopentenyladenine (2iP). The use of antibodies in rapid tests to analyse hormones has been developed for the immediate application of high sensitivity immunoglobulins against IAA, 2iP and Z. Each plant hormone was conjugated to bovine serum albumin (BSA) or ovalbumin (OVA), both of animal origin, using chemical couplings to their respective functional groups. The IAA, Z and 2iP antibodies were then produced from chicken egg yolks (IgY) or from rabbit serum (IgG) that had been hyperimmunised with the respective conjugated protein. The antibodies were obtained from rabbit sera or chicken egg yolks, purified by ammonium sulphate precipitation and subsequently used in indirect analytical immunoassays (competitive enzyme-linked immunosorbent assay; ELISA) to detect and quantify these hormone molecules. During titration, differences were observed not only between the specific antibody sources but also between the different conjugated hormones obtained. Although, the IgGs failed to produce a competitive IAA detection system, the IgY antibodies proved to be suitable for quantifying the plant hormones. The immunoenzymatic assay using IgY achieved a detection limit of 3.4 nmoles (3.4 ppb) for zeatin, 6 pmoles (6 ppt) for 2iP and 480 pmoles (480 ppt) for IAA, whereas the IgG detection limits established were in micromolar (ppm) range and manifested varying results and low specificity near the limit of detection. Comparisons and discussions are presented on the impact of the hormone-protein conjugates and the potential use of antibodies in analysing zeatin and 2iP. The antibody characteristics, the presence of antibody interference in the samples and the limitations and advantages of immunochemical assays are also discussed.

Key words: Quantification, auxin, cytokinins, antibodies..

INTRODUCTION

Immunochemistry emerged in the 1960s, nearly one hundred years after the pioneering studies of Paul Ehrlich on the immunological quantification of ricin and abrin (Silverstein, 2002), and evolved into the development and

*Corresponding author. E-mail: sousacm@yahoo.com.br or rmottam@ufrrj.br.

validation of quantitative immunoassays that have emphasised the affinity, sensitivity and selectivity of antibodies (Berson and Yalow, 1968). The practical limiting properties of immunoassays (IAs) have allowed for the detection of small yet specific structures, which are widely applied as tools in therapies, diagnostics and research in food technology science and in pharmaceutical, forensic, environmental, veterinary and agronomic analyses. The need to understand and elucidate the signalling, function and dynamics of hormones has increased the demand for efficient, high sensitivity and low cost analytical methods for detecting hormone molecules (Izumi et al., 2009). The analytical methods commonly used to study the control and regulation of plant growth are based on the use of chromatographic techniques [for example, gas chromatography-mass spectrometry (GC/MS) and high-performance liquid chromatography-mass spectrometry (HPLC/MS)], which in most cases do not achieve the necessary limits of detection, require a significant time commitment for each analysis and require advanced preparation of each sample to concentrate the analyte and purify the preparations (Izumi et al., 2009; Toker et al., 2006).

Faced with these drawbacks, immunochemical techniques allow for the direct and simultaneous analysis of numerous samples in a simple and rapid manner because the major features of these techniques are a high detection capacity, specificity and rapidity (more than one hundred samples can be processed simultaneously) (U.S. NRC, 1992; Nunes, 2005). Immunochemical techniques are fundamentally based on the use of specific antibodies to detect particular compounds. The methods used to detect this molecular recognition reaction give rise to a variety of immunochemical techniques, such as immunoassays, including enzyme-linked immunosorbent assays (ELISA), immunoaffinity chromatography (IAC) and immunosensors (Mikrova et al., 2003). The low cost and ease of use of immunochemical techniques make them accessible and promising methods for determining the levels of endogenous hormone molecules in plants by analysing the structural components of plant hormones (Maldiney et al., 1986). These techniques can thus significantly contribute to the understanding of plant hormone dynamics within plant tissues.

Plant hormones are organic molecules that at low concentrations induce certain physiological responses, thus controlling the growth and development of plants. Among the classes of hormones, auxins, cytokinins, gibberellins, abscisic acid and ethylene are notable. Auxins are considered to be responsible for cellular division, cellular differentiation and cellular expansion (Schenck et al., 2010), whereas cytokinins are responsible for cellular division, chloroplast development, bud differentiation, the initiation and growth of shoots and the growth and senescence of leaves (Mok and Mok, 2001). In 1893, klemperer demonstrated that the immunisation of chickens resulted in the transfer of specific antibodies from the blood into the yolks of their eggs. Eighty-seven (87) years later, with the increasingly widespread ethical concerns about the welfare of experimental animals, this method for obtaining antibodies without sacrificing the animals began to draw more attention. This technique is currently implicated in a wide range of analytical and experimental possibilities to develop new IAs. Recently, several immunochemical methods for detecting multiple biomarkers and environmental and biological fluid contaminants have been developed based on IgYs (U.S. NRC, 1992; Freire et al., 2004; Nunes, 2005).

Greunke et al. (2008) showed advantageous behaviour of monoclonal IgY as detection or capture antibodies compared to conventional mammalian immunoglobulins and provide a strategy for improvement of assay performance and accuracy. CIgY-ELISA is an immunochemical technology which could be used in agronomic studies for a better understanding of aspects of the growth and development of the plants, and in their interactions with the environment and studies such as plant domestication. The IgY-based analysis presented here suggested that the changes in the plantlet architecture and development were consistent with the reduction of cytokinins (Sousa et al., 2011).

The present study aimed to obtain rabbit IgG and chicken IgY antibodies and compare them in plant hormone analyses using immunoenzymatic assays to determine the levels of IAA, Z and 2iP molecules.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits weighing approximately 2.0 kg and young laying ISA-Brown (Hy-Line Brown) hens that had just begun to lay eggs were used. The animals were kept in climate-controlled rooms in appropriate individual cages, and drinking water and food were provided *ad libitum.*

Reagents and instruments

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). The dialysis bags were made from Spectra/Por Spectrum membranes (Houston, TX, USA) (MWCO: 12 to 14 kDa). The electrophoretic characterisation was performed using a Mini-Protean II apparatus (BIO-RAD Laboratórios Brasil Ltda, Rio de Janeiro). The ELISA assays were performed in 96-well serology microplates (flat bottom) (Nunc, Roskilde, DK, #442404) and the optical density (OD) measurements were taken in an ELISA reader (microplate reader).

Synthesis and conjugation of haptens

The conjugation reactions were performed according to methods previously described in the literature. The conjugation of indoleacetic acid (IAA) was performed by the chemical activation of side groups on each hapten using an ambiguous spacer, followed by coupling to the carrier protein, as described by Maldiney et al. (1986) and Sousa et al. (2011). The conjugation occurred in the presence of N-N-dimethylaminopropyl-N-ethylcarbodiimide (EDPC)

hydrochloride. A solution containing 1.0 mM IAA was added dropwise to a solution of 31.74 mM carrier protein [bovine serum albumin (BSA) or ovalbumin (OVA)] under constant stirring at room temperature. The pH was adjusted to 8.3 with 0.1 N NaOH, and the final volume was 10 ml. The IAA-carrier coupling was generated by adding 5.02 mg EDPC at four 2-h intervals at 4°C under constant stirring in the dark. The zeatin (Z) and isopentenyladenine (2iP) conjugates were generated by activating the riboside fraction present on the structure or via their side chains such as the isopentenyl side chain present on trans-zeatin (Weiler, 1980). Ten millilitres (10 ml) of 0.01 M sodium periodate was added dropwise for every 20 mg of cytokinin in 4 ml of methanol. The mixture was stirred (Vortex) for 20 min, and the excess periodate was removed by adding 0.6 ml of an aqueous solution containing 0.1 M ethylene glycol.

The mixture was then homogenised for an additional 5 min to be coupled to the carrier protein. Meanwhile, the BSA (1.7 mM) and OVA (1.5 mM) solutions were prepared. The pH of each protein solution was adjusted to 9.3 by adding an aqueous solution of 5% K_2CO_3. The reactive hapten solution was divided into two aliquots and added to each of the protein solutions, while the pH was carefully maintained between 9.2 and 9.4. The solutions were then homogenised at room temperature for 1 h. The pH was adjusted to 6.5 with an acetic acid solution (1 M), and the mixtures were again stirred for two additional hours. The resulting solutions containing the desired conjugates (10 ml) were dialysed at 4°C against 300 ml of distilled water for 48 h at 18-h intervals. The dialysed conjugates were divided into 1-ml aliquots and stored at -20°C for subsequent use. The protein-2iP conjugates were prepared in a manner similar to the Z conjugates.

Immunisation

The inocula were prepared from a mixture of 1.0 ml of the conjugate and 1.0 ml of Freund's complete adjuvant. The immunisations consisted of 0.1 ml inoculations of this mixture into the backs of the rabbits at 15 to 20 points via intradermal injection (Li et al., 2003) or into the pectoral muscles of chickens at 10 points via intramuscular injection (Munene, 2005; Freire et al., 2004). The rabbits and birds received three 500-µL boosters of the mixture, consisting of 1.0 ml of the conjugate and 1.0 ml of Freund's incomplete adjuvant. The rabbits were boosted at weeks 1, 2 and 5 following the initial inoculation, whereas the chickens were boosted every three weeks.

Obtaining antibodies

IgG antibodies were obtained from 50 ml of rabbit blood via cardiac puncture. The blood was placed in a sterile glass vial (200 ml), capped with aluminium foil and maintained at room temperature for 2 h. The serum was then centrifuged at 600 *g* for 10 min at 4°C. The precipitate was discarded, and the supernatant was transferred to a glass vial and stored at 4°C overnight. The clot that was formed was removed from the walls of the vial and stored overnight at 4°C. The following morning, the released serum was centrifuged under the same conditions as aforementioned and mixed with the serum obtained the previous day. The IgY antibodies were obtained by diluting egg yolks in ice water (4°C) at a 1:10 dilution. The final egg yolk solution was homogenised for several minutes, and the pH was adjusted to 5.0. The sample was kept at 4°C overnight and then centrifuged at 10,000 g for 30 min at 4°C. The supernatant, containing IgY and other soluble proteins was separated from the precipitate and transferred to a clean container (Munene, 2004).

Precipitation of globulins

The IgGs were precipitated by adding an equal volume of saturated $(NH_4)_2SO_4$ (5.35 M) at 4°C under stirring at room temperature. The solution was stored for 1 h at 4°C and centrifuged at 10,000 *g* for 20 min. The precipitate was diluted with a 50% saturated ammonium sulphate solution (1:4) and stirred slowly for 30 min. The solution was then centrifuged, and the precipitate was suspended in phosphate buffered saline (PBS) buffer (pH 7.2). The IgY precipitation involved preparing a solution containing saturated ammonium sulphate and yolk diluted (1:1), which was subsequently stored overnight at 4°C. The mixture was then centrifuged at 10,000 *g* for 20 min at 4°C. The resulting precipitate was dissolved in saline solution (0.15 M) and centrifuged for 10 min at 10,000 g at 4°C. The resulting total precipitate from the egg yolk was diluted 1:10 in PBS buffer (pH 7.2). The solutions containing the IgY or IgG antibodies were dialysed against distilled water for 72 h with two daily water changes and subjected to further purification using low pressure liquid chromatography (LPLC) in a molecular sieve gel (Sephadex G25, Pharmacia) using 0.01 M PBS (pH 7.2) as an eluent.

Antibody characterisation by electrophoresis

The electrophoresis characterisation mixtures consisted of 30 µl of IgG or IgY and 970 µl of a solution containing 2% sodium dodecyl sulphate (SDS), 14.4 mM 2-mercaptoethanol, 25% glycerol and 0.1% bromophenol blue dissolved in 60 mM Trizma base, pH 6.8. The samples were heated to 100°C for 5 min in a water bath. Electrophoresis was performed in running buffer (25 mM Trizma base, 192 mM glycine and 0.1% SDS, pH 8.3) at 40 V for stacking and 100 V for separation. The molecular weight marker M4038 (Sigma) was used to calculate the estimated molecular weight of the separated peptide fractions. The gels were fixed and stained with 0.25% Coomassie blue (Harlow and Lane, 1988).

Enzyme-linked immunosorbent assay (ELISA)

A titration was performed in 96-well ELISA plates containing eight rows and 12 columns. The antibodies were diluted along a row and the conjugates along the columns as described by Crowther (1995) and Peres et al. (1997). In the rabbit-derived antibody titrations, anti-IgG antibodies were used, and anti-IgY antibodies were used for the antibodies derived from the chicken egg yolk. For detection, the plates were placed at 37°C for 1 h, washed with PBS + 0.05% Tween and dried. Finally, 200 µl of ortho-phenylene diamine (OPD) substrate was added and incubated for 20 min in the dark at room temperature. The reaction was stopped with concentrated sulphuric acid, and the optical densities were then read. From the titration, the optimal concentrations of conjugate and antibody were defined, and competitive ELISAs were performed to determine the limits of detection of the haptens for the antibodies produced. The competetion process consisted of sensitising the plates with the optimal concentrations of conjugants, as defined in the previous step (titration).

Simultaneously, 100 ml of PBS buffer was added to all the wells of another ELISA plate, and 100 ml of a solution containing 50 nM hapten was added to four replicate wells in the first column. Twenty (20) serial dilutions were performed from this initial concentration, yielding concentrations that ranged from 50 nM to 0.1 pM. The specific antibody against the molecule of interest was then added at the concentration determined by titration and incubated at 37°C for 2 h. The mixture containing the hormone solution and the antibody was then transferred to the plate, which had been sensitised with carbonate/bicarbonate buffer for 2 h at 37°C, washed with PBS + 0.05% Tween 20 and dried. The detection process was as described for the titration.

RESULTS AND DISCUSSION

Immunising laying hens and rabbits with the IAA, Z and

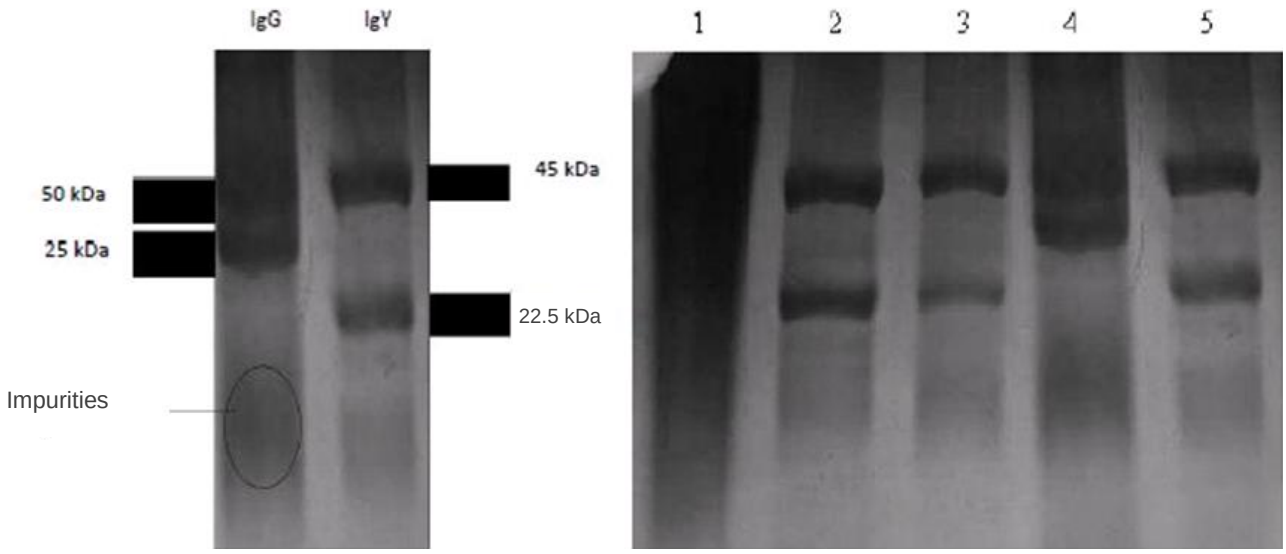

Figure 1. Polyacrylamide gel (10% SDS-PAGE) of IgG and IgY samples against indoleacetic acid (IAA) and cytokinin (2iP). Lanes: 1 – anti-IAA IgG; 2 and 3 - anti-IAA IgY; 4 - anti-2iP IgG; 5 - anti-2iP IgY. This gel shows the separation of the Fc (heavy chain) and Fab (light chain) fractions of the antibodies and the presence of impurities in the preparations. The running conditions are described in the materials and methods.

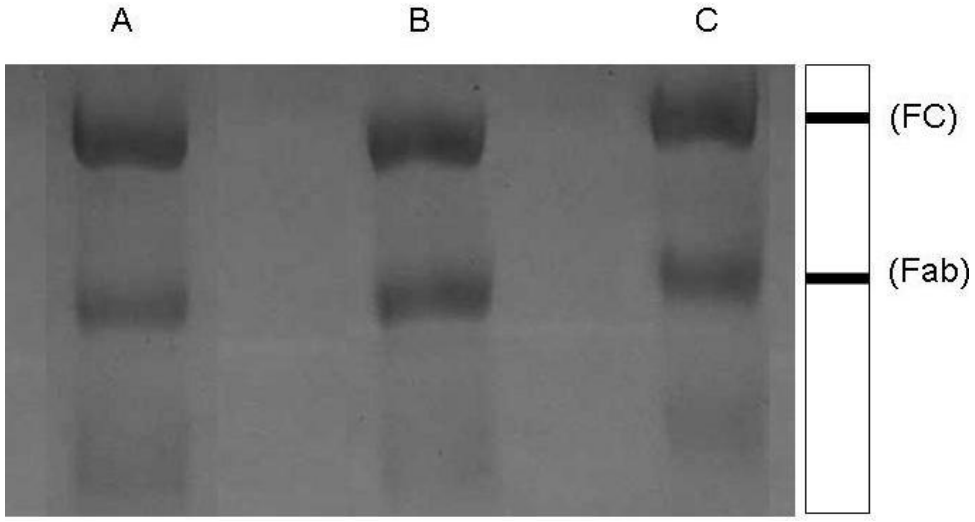

Figure 2. Polyacrylamide gel (SDS-PAGE) showing the separation of the Fab and Fc fractions and the impurities in the IgY antibody samples against IAA (A), zeatin (B) and 2iP (C) obtained from chicken egg yolks.

2iP conjugates enabled us to obtain antibodies against these hormone molecules. Studies were performed to compare these antibodies to determine their potential use as immunochemical tools in the analysis of plant hormones. The purification of antibodies from the chicken egg yolks enabled us to obtain antibodies with greater purity compared to antibodies from the rabbit sera (Figure 1), confirming the greater practical potential of IgY preparations compared to rabbit IgG. The electrophoretic analysis of the antibodies (IgG and IgY) showed that the IgY purifications were more efficient than the IgG purification (Figure 1) because interfering proteins were not present, resulting to the sharp detachment of the two chains. The molecular mass of IgY was estimated to be 180 kDa, of which, the FAB_{IgY} fraction corresponded to 45 kDa (22.5 kDa for each Fab'_{IgY} fraction), and the FC_{IgY} fraction corresponded to 135 kDa (67 KDa per FC_{IgY} monomer). The rabbit IgG had a molecular mass of 150 kDa, corresponding to 50 kDa for the Fab_{IgG} fraction (25 kDa for each FAB'_{IgG}) and 100 kDa for the FC_{IgG}, which is consistent with the literature (Shimizu et al., 1993) (Figures 1 and 2). Figures 3 and 4 show the variations in

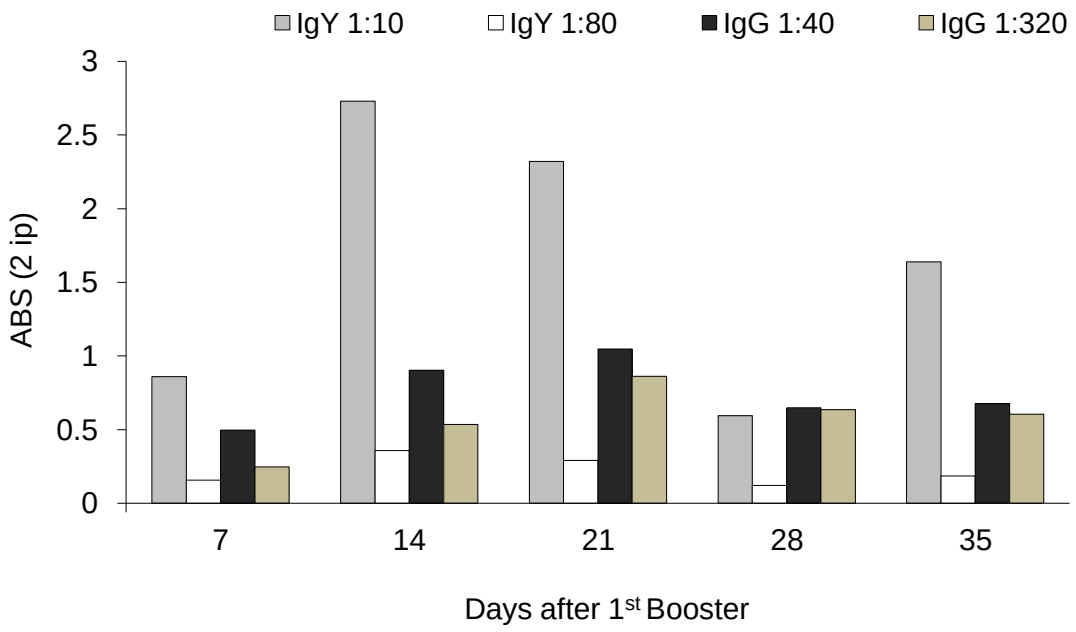

Figure 3. Relative concentrations of specific IgY and IgG antibodies against cytokinin (anti-2iP) recovered after the first booster. Each bar represents the average of four replicates.

the relative concentrations of anti-2iP and anti-IAA antibodies produced in rabbits and chicken egg yolks after the animals received booster immunisations. Com-paring the absorbances of the anti-2iP IgY and IgG antibodies indicated that IgY exhibited the highest absor-bance on day 14 following the first booster, whereas the IgG was highest on day 21 following the first booster (Figure 3). For the anti-IAA, the peak was observed on day 14 for IgG (at a 1:40 dilution), whereas IgY (at a 1:10 dilution) peaked on day 28 following the first booster (Figure 4).

Different dilutions were adopted for each species, the specifics of which were determined by the ELISA titrations for each antibody. Anti-zeatin antibodies were only obtained from the chicken egg yolks. Although, there were variations in the intensity of the responses against the different target analytes among the immunised birds, the antibody production response against these hormone molecules seemed to be dependent on the animal used and the ideal time to purify the antibodies, despite the variation due to the molecule studied (Figures 3 and 4). These results are consistent with the literature, which outlines five basic factors that are capable of influencing the responses of birds to immunisation: antigen (dose and molecular weight), the type of adjuvant used, the route of immunisation used, the frequency and the interval between the different immunisations (Schade et al., 2000). The adopted immunisation procedure induced an antibody response 14 days after conjugate inoculation, with the maximal peak at approximately 35 days post-inoculation. The lipid fraction, including triglycerides, phospholipids and cholesterol, comprised 30 to 40% of the egg yolk. The protein fraction represented, on average, 16% of the egg yolk.

The IgY fraction comprised approximately 14% of the soluble proteins obtained after delipidation of the egg yolk, ammonium sulphate precipitation and filtration chromatography over a Sepharose (G25) gel, comprising approximately 2.24% of the egg yolk content. Thus, at the end of the immunisations, an average of 112 mg IgY could be obtained per 4.5 g of egg yolk from hyperimmunised chickens, which is consistent with the literature (Munene, 2004). Considering that a laying hen can produce approximately 25 eggs per month, the estimated amount of antibodies per immunised bird is between 2.8 and 5.6 g per month. This amount is unsurpassed compared to the amount that can be obtained from the successive bleeding of a rabbit (Schade et al., 2000). Wolley and Landon (1995) reported that antibodies obtained from sheep had a higher titre compared to antibodies obtained from chickens, independent of the time period following immunisation. This characteristic is likely related to the molecular structure of IgY compared to mammalian IgG. The heavy chain of an IgY molecule contains a variable domain (V_H) and four constant do-mains (C_H1, C_H2, C_H3 and C_H4), whereas the mammalian IgG heavy chain is comprised of three constant domains (C_H1, C_H2 and C_H3). In the IgG heavy chain, the C_H1 and C_H2 domains are separated by a proline-rich region called the hinge region which provides a high level of flexibility and therefore an increased ability to form mole-cular interacttions; because this region forms the base of the Fab fragment, there is greater potential for interaction between antigen and antibody.

IgY, on the other hand, is devoid of this hinge region

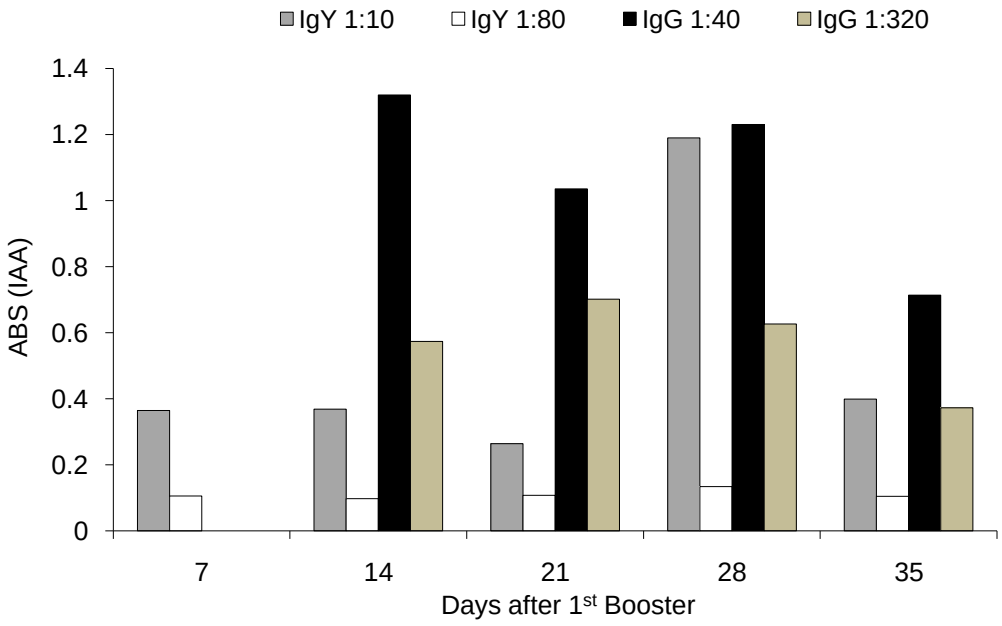

Figure 4. Relative concentration of specific IgY and IgG antibodies against auxin (IAA) recovered after the first booster. Each bar represents the average of four replicates.

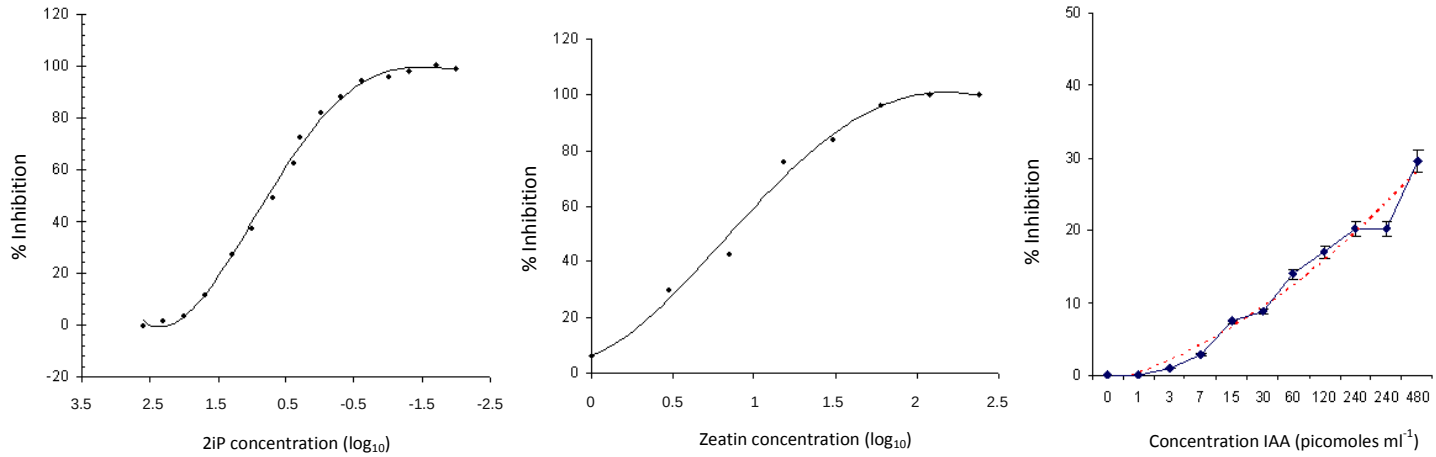

Figure 5. Competition curves of the hormone molecules using IgY antibodies.

but has a high concentration of glycine and proline residues between the C_H1-C_H2 and the C_H2-C_H3 linkage domains that do not interact with the antigen. Thus, IgYs are neutralising antibodies that interact with antigens in a more limited manner than the mammalian IgGs. Furthermore, IgY, unlike IgG, is devoid of intracatenary disulphide bonds that link the variable and constant portions of the light chain, resulting in reduced stability and therefore, weaker antigen bonding compared to mammalian IgG (Shimizu et al., 1993). An inhibition curve was generated by reacting to the antibodies at the dilutions indicated by the titration with various concentrations of the hormone molecule (Figure 5). Therefore, we attempted to establish response curves in sigmoid plots with a central linear range. According to Nunes (2005), when the concentration of the molecule is too low, the equilibrium favours a high concentration of enzyme conjugate bound to the antibody, and the corresponding absorbance will be the highest. The working range of the calibration curve is bounded by upper and lower limits. Within this range, the variation in the absorbance is related to the concentration of the analyte. At higher concentrations (upper limit), the assay is saturated, and an increase in the analyte concentration has no effect. The working range of this type of curve is an important feature and provides the first clue to the sensitivity of the test.

The competition analyses comparing the anti-IAA antibodies produced in both rabbits and chickens were compromised. We could not establish an IC50% for this molecule, but an IC near 30% was determined, which was approximately 480 pmoles. The concentration limits for linearising the response to IAA (from 7.0 pmoles) were consistently higher than those obtained for zeatin (between 3.4 and 11 nmoles) and 2iP (6 and 150 pmol) (Figure 5).

Conclusion

The immunisation of laying hens and rabbits with synthesised conjugates resulted in the production of specific antibodies against hormone molecules. The antibodies obtained from both rabbits and chicken egg yolks exhibited excellent potential for use in immuno-chemical tests for analysing plant hormones. The IgY obtained using ammonium sulphate precipitation showed greater potential for use in immunochemical assays than the obtained IgG.

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