

Application of Plantibodies, the plant-made vaccines

Abstract

Various approaches are used to integrate the desired genes encoding the antigen protein for a given illness into the genome of plant tissues in plant-based vaccination technology. Gene transfer by agrobacterium and transformation via a genetically engineered plant virus are two typical approaches for producing efficient vaccinations. Antibodies are an important component of vertebrates' adaptive immune systems, and they may now be made by converting plants with antibody-coding genes from animals and humans. Despite the fact that plants do not produce antibodies naturally, plant-derived antibodies (plantibodies) have been proven to behave similarly to mammalian antibodies. However, as science and technology have progressed, new approaches have been created to improve the efficiency of older technologies including biolistic, electroporation, agroinfiltration, sonication, and polyethylene glycol treatment. Despite the fact that plant-based vaccinations have numerous advantages for the vaccine industry, there are still constraints that limit the rate at which these third-generation vaccines may be successfully manufactured. Despite these limitations, continued attempts are still underway to develop effective vaccines for a variety of human and animal diseases, owing to its enormous potential.

Keywords: Plantibodies, Transgenic, Phytopharming, Therapeutics etc.,

INTRODUCTION

Earlier in time most medicinal compounds came from plants. Plants are the raw source materials for as many as 40% of the pharmaceuticals in use. Some commercial medicines, such as the cancer drugs taxol from *Taxusbrevifolia*, colchicines from *Gloriosasuperba*, camptothecin from *Camptothecaacuminata*, ginsenosides from *Panax ginseng*, vinblastine from *Catharanthusroseus*, the anti-malarial drugs quinine from *Cinchona pubescens*, and artemisin from *Artemisia annua*, are manufactured from plants. But beginning about 50 years ago, chemistry took over from botany, with most new drugs being artificially

produced in pharmaceutical labs (Moffat, 1995). Nowadays, one of the most promising methods of producing proteins and other medicinal substances, such as antibodies and vaccines, is the use of transgenic plants.

PHYTOPHARMING

A transgenic plant contains a gene or genes that has been artificially inserted. The inserted gene sequence, known as the *transgene*, may come from an unrelated plant, or a completely different species. One of the purposes of inserting a combination of genes in a plant is to make it as useful and productive as possible. This process provides advantages such as higher yield, improved quality, pest or disease resistance, and tolerance to heat, cold and drought. However, transgenic plants can also be produced in such a way that they express foreign proteins with industrial or pharmaceutical value. Transgenic plants represent an economical alternative to fermentation-based production systems. Plant-made vaccines or antibodies (*plantibodies*) are especially striking, as plants are free of human diseases, thus reducing screening costs for viruses and bacterial toxins (Herbers *et al.* 1999). The first transgenic plants were reported in 1983. Since then, many recombinant proteins have been expressed in several important agronomic species of plants including tobacco, corn, tomato, potato, banana, alfalfa and canola (Hammond *et al.* 1999). The choice of plant systems was initially driven by convenience and the need to evaluate genetic constructs quickly.

The use of plants as bioreactors, or molecular farming, is a technology comprising both, the expression and characterization of recombinant proteins in plant hosts and its high scale production (host plant cultivation, harvesting and biomass storage, processing and purification of the protein of interest, and its related quality control processes and regulatory issues). Using plants as a bioreactor for the production of recombinant proteins have advantages and disadvantages compared with other expression systems such as animal systems, bacterial systems, yeast systems and etc. The most important disadvantage of prokaryotic systems is their inability to perform post-translational modification of produced proteins. Antibodies produced by the plants have been affinity absorption and ability of specific bind to antigen similar to hybridoma cell products and as well as, their properties will be maintained after purification. Many of the early, plant-derived recombinant proteins were produced in transgenic tobacco plants. The continuing popularity of tobacco reflects its status as a well-established expression host for which robust transformation procedures and well-characterized regulatory elements for the control of transgene expression are available.

Furthermore, its high biomass yields and rapid scalability make tobacco very suitable for commercial molecular farming. It is also a non-food, non-feed crop, and carries a reduced risk of transgenic material or recombinant proteins contaminating feed and human food chains (Stoger et al., 2000). Tobacco has been adopted as a platform system by several biotech companies, including Planet Biotechnology Inc. (<http://www.planetbiotechnology.com/>) and Meristem Therapeutics (<http://www.meristem-therapeutics.com/>), only two companies to have plant-derived pharmaceuticals undergoing phase-II clinical trials. One disadvantage of tobacco is its high content of nicotine and other toxic alkaloids, which must be removed completely during downstream processing steps. Although low-alkaloid tobacco cultivars are available, attention has turned to other leafy crops for pharmaceutical production. These crops include lettuce, which has been used for clinical trials with a hepatitis B virus subunit vaccine, and alfalfa, which is being promoted as a platform system by Medicago Inc. (<http://www.medicago.com>). Proteins that are expressed in cereal seeds are protected from proteolytic degradation, they can remain stable for up to three years at room temperature (E Stoger, unpublished data) and for at least three years at refrigerator temperature without significant loss of activity (Larrick and Thomas, 2001). Several different types of cereal, including rice, wheat, barley and maize, have been investigated as potential hosts for recombinant protein production. Potatoes have been evaluated for the production of human serum albumin, novel vaccine candidates, tumour necrosis factor α (TNF- α) and antibodies (Dewilde, *et al.*, 2002). Other production hosts that have been used to express vaccines include tomatoes, bananas, carrots, lettuce, maize, alfalfa, white clover and Arabidopsis. Oil crops are useful hosts for protein production because the oil bodies can be exploited to simplify protein isolation. An example is the oleosin-fusion platform developed by SemBioSys Genetics Inc. (<http://www.sembiosys.com/>), in which the target recombinant protein is expressed in oilseed rape or safflower as a fusion with oleosin. Finally, there have been significant recent developments in the use of more diverse plant species, which can easily be contained, propagated and transformed, to produce recombinant proteins. Mayfield *et al.* (2003) have described a protein expression system that is based on the unicellular green alga *Chlamydomonas reinhardtii*. In this system, chloroplast-targeted transgenes were used to express an antibody that recognised herpes simplex virus glycoprotein D. Other simple plants that have been adopted as bioreactors include Lemna (duckweed), which is being developed as a platform technology by Biolex Inc. (<http://www.biolex.com>); and the moss *Physcomitrella patens*, which is being developed by Greenovation Inc., Freiburg, Germany (<http://www.greenovation.com>).

PLANTIBODIES

Antibodies and glycoproteins (including therapeutic proteins) expressed in plants require glycosylation which is essential for stability, solubility, proper folding and biological activity (Samyn – Petit, 2003). The covalent linkage of an oligosaccharide side chain to a protein is known as “glycosylation”. In most glycoproteins, the oligosaccharide side chain is attached to the amide nitrogen of an asparagine (Asn) residue (termed N-glycosylation) and/or to the hydroxyl of threonine (Thr), serine (Ser) or hydroxyproline (Hyp) (termed O-glycosylation) residues of the peptide backbone. These processes represent the most widespread post-translational maturation (PTM) found in natural and biopharmaceutical proteins. In plant cells, as in any other eukaryotes, N-glycosylation starts in the lumen of the endoplasmic reticulum (ER) by the cotranslational transfer of an oligosaccharide precursor, Glc3Man9GlcNAc2, from a Dolichol (which anchors the oligosaccharide at the ER membrane) onto specific Asn residues constitutive of the N-glycosylation sequences, Asn-X-Ser/Thr. While the basic N-acetyl glucosamine (GlcNAc)-mannose precursor structures added to the glycosylation sites of proteins in the endoplasmic reticulum of plant and mammalian cells are identical, further maturation in the Golgi apparatus brings about important variations in plant protein glycosylation compared to the mammalian cells. Some of these important differences are: in the case of plantibodies, the addition of

- (i) a (1,3)-fucose (instead of a(1,6)-fucose) to GlcNAc,
- (ii) b (1,2)-xylose (non-existent in mammalian cells) to mannose and
- (iii) b (1, 4)-fucose and b (1, 3)-galactose (instead of b (1, 4)-galactose) to GlcNAc (Gomorod et al., 2004).

These clearly indicate that, if a mammalian glycoprotein is expressed in plants, it will have a different glycan structure caused by the differences in plant and human N-glycan processing. Apart from the principal possibility that the function of an incorrectly glycosylated protein can be lost or altered (e.g. due to misfolding), another problem is that the core Xyl and core á (1, 3), Fuc epitopes are known to be important IgE binding carbohydrate determinants of plant allergens.

The most important requirements for the production of heterologous proteins are genes or cDNAs encoding the desired proteins, a suitable vector and an expression system which is able to appropriately produce the desired protein. Once the appropriate cDNA encoding the desired protein is isolated from a hybridoma cell line or through phage display library, it is inserted into a vector designed for high expression levels that maximise the rates of transcription and translation. The choice of vector and promoters depends, in general on

needed requirements and optimized vectors are commercially available. Since the expression of a foreign gene is frequently regulated at the level of transcription, boosting the rate of transcription increases protein yield. This can be achieved at the level of transcription initiation, RNA processing and RNA stability. Deleting or modifying synthesized RNA transcripts which usually have a specific recognition site that shortens the RNA half-life can also increase protein yield (Desai *et al.*, 2010). Constitutive promoters have been shown to produce higher expression levels than inducible, regulated or tissue-specific promoters. In this case, the proteins are expressed in other vegetative tissues of the plant which may negatively affect its growth and development. However, to minimize the toxic effect of recombinant proteins on the plants as well as other life forms that may have contact with them as well as its leaching into the environment it is much safer to use regulated and inducible promoters.

PROMOTERS

In dicotyledonous species, the strong and constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter is often used to drive transgene expression while in monocot (cereals) the maize ubiquitin -1(*ubi-1*) promoter is preferred due to the lower activity of CaMV35S. The constitutive promoter of viral origin can result in gene splicing via co-suppression which is not common in the constitutive promoter of plant origin. Certain synthetic promoters are developed for maximising expression levels and are made by combining the most active sequences of multiple well characterised natural promoters. The insertion of introns, a phenomenon known as intron-mediated enhancement also increases the rate of transcription in cereals (Twyman *et al.*, 2003).

Other strategies to boost expression levels involve the use of multiple copies of enhancer sequences from a highly active promoter, the addition of scaffold attachment sequence or matrix attachment regions next to promoters and the stacking of transcription units. Co-expression of foreign protein with protease inhibitors is a promising strategy which can minimize the foreign protein degradation in plant tissues. Additionally, co-expression with antigen has been shown to improve the expression of antibody which may be as a result of the stabilization of antibodies by the binding antigen (Stoger *et al.*, 2002).

GLYCOSYLATION

One of the most drastic approaches for the humanization of plant-made glycoproteins is to prevent the addition of immunogenic N-glycans to the polypeptide. This can easily be achieved by mutating the nucleotide sequence (and thereby the amino acid) of the final product in a way that a potential N-glycosylation signal sequence (Asn –X – Ser/Thr) is lost.

This strategy in most cases, will not inactivate the antibody's antigen binding function. However, many pharmaceuticals, including antibodies for their effects or functions, (such as the triggering of the immune response) require glycosylation for an increase in vivo activity and stability (Gomordet *al.*, 2004). Additionally, it has been discovered that both proteins can be increased by the addition of N-glycans. This further illustrates the possibility, in glycol engineering, to increase the number of glycosylation sites on recombinant pharmaceuticals. Finally, by avoiding glycosylation processes, the term “humanization” must be put in perspective: not a human-like glycan structure, but achieving an avoidance of human immune responses.

CHALLENGES

The two major challenges that still limit the cost-effective production of plant-made recombinant proteins are inadequate protein accumulation levels and the lack of efficient as well expensive purification methods (Joensuu *et al.*, 2010). Fusion proteins have been developed for the purpose of affinity purification of recombinant proteins (Nilsson *et al.*, 1997). The recombinant proteins are fused translationally to small affinity tags or to proteins with defined binding characteristics these fusion proteins can then be purified from the crude biological using a single step, fusion partner-specific and affinity chromatography step. The use of affinity tags to facilitate the recovery of protein is a useful technique, as long as the tag can be removed after purification to restore the native structure of the protein (Fischer *et al.*, 2004). However, the purity and recovery of most of these recombinant proteins are low using some fusion partners. Oleosin-fusion platform is a system developed by SemBioSys Genetics in which the target recombinant protein is expressed in oilseed crops as a fusion with oleosin. The defunct Finnish biotech company UniCrop also developed an oilseed technology platform which isolates recombinant proteins from the rapidly developing sprouts cultivated in bioreactors. The fusion protein can then be recovered from oil bodies using a simple extraction procedure and the recombinant protein is separated from its fusion partner by endoprotease digestion (Moloney *et al.*, 2003). Degradation of abnormal or incorrectly processed proteins is ubiquitous in nature. While some proteins accumulate at high levels in plant systems, other proteins apparently undergo extensive hydrolysis, with a strong negative impact on the final yield despite easily detectable mRNA transcripts. Accumulation levels below 0.01% of total soluble protein (TSP) in plant tissues have been observed for several proteins of therapeutic value, including, for instance, human serum protein C, interferon α , erythropoietin and epidermal growth factor (Daniell *et al.*, 2001). Low accumulation rates of intact protein products have also been observed for other recombinant proteins of practical

interest, including immunoglobulins, antibodies and enzyme inhibitors, again suggesting the key influence of proteolysis on the overall efficiency of plant-based protein factories. However, proteolytic degradation of foreign proteins can be minimised by targeting to the secretory pathway. The plant cell endoplasmic reticulum (ER) contains a few proteases and as such proper post-translational modification and assembly of foreign protein can also occur in the ER. Certain ER retention signal sequences like KDEL and HDEL are also used to target protein synthesis to the ER. Expressing recombinant protein in certain organs such as the seed and tuber can minimize its degradation. These organs usually contain endogenous protease inhibitors that provide protection from proteolytic attack. These are some of the necessary factors to be considered in planning the production of antibodies and other therapeutic proteins in plants. Several strategies have been considered to minimize proteolysis in plant protein factories.

The lack of expression of the inserted transgene to produce the coded proteins in plants is one of the problems that may occur after transformation events. The two types of gene silencing observed in plants are transcriptional gene silencing (TGS) and post-transcriptional gene splicing (PTGS) (Hernandez-Pinzon *et al.*, 2007). Silencing seems to be strongly associated with the copy number of transgene present as well as expression level. There are several ways in which gene silencing can be overcome: (1) by the use of *Agrobacterium tumefaciens* which results in fewer copies than biolistic transformation (Hansen *et al.*, 1997). (2) flanking transgenes with matrix attached regions (MAR) sequences (Allen *et al.*, 2000) increase the expression of transgenes and may also reduce silencing (3) the use of viral suppressors of silencing can be used to prevent or reverse PTGS. The rate of protein synthesis can be optimized by making sure that the translation start site conforms with the Kozak consensus for plants and replacing any native untranslated sequence with translational enhancers such as the 5' leader sequence derived from Tobacco Mosaic Virus (TMV) RNA (the omega sequence).

To achieve specific antibody and therapeutic protein production in plants, the DNA that encodes the desired protein has to be first inserted into the plant cell. The two major transformation techniques are stable transformation and transient expression. Stable nuclear transformation, the incorporation of foreign gene or gene(s) into the nuclear genome of the plant, has produced most of the recombinant proteins to date. A major disadvantage of stable nuclear transformation is the random insertion of the transgene into the genome which continues to make it imperative to screen transform plants to define the position of transgenes and evaluate their copy number within the plant nuclear genome. In plant cells, it is also possible

to deliver genes of interest into chloroplast and mitochondria; because plants have multiple copies of chloroplast per cell, chloroplast transformation has the potential for high expression levels and consequently high yield of recombinant proteins. Chloroplast transformation makes use of two flanking sequences, which during homologous recombination insert foreign DNA into the spacer region between the functional genes of the cont..

chloroplast genome. Since genes are targeted to a precise location, this eliminates the chances of its insertion in a different location (a phenomenon known as “position effect” frequently observed in nuclear transformation); thus preventing “gene silencing”. However, its prokaryotic origin may pose problems during post-translational modifications of complex proteins. Plant cell cultures can also be used for the production of recombinant proteins. The cells are first stably transformed with the antibody transgene, the process is very rapid as regeneration is not required and yields of up to 25 mg/l of recombinant antibody are possible. Extraction and purification and containment of recombinant antibodies are also easier.

Rhizosecretion, a method using root cultures, is also an alternative approach to cell culture for the generation of mAb? Full name monoclonal antibody? in transgenic plants (Koet *et al.*, 2005). The transient transformation includes agroinfiltration and transduction. Transduction is the use of recombinant plant viruses to deliver genes into plant cells (Thomas *et al.*, 2002). The two-host virus system most frequently used were tobacco with tobacco mosaic virus (TMV) and cowpeas with cowpea mosaic virus (CPMV). The DNA coding for the desired protein is engineered into the genome of the plant virus that will be used to infect the host plant. As the virus replicates and spreads within the plant, many copies of the desired DNA will be produced and high levels of protein production are achieved within a very short time, and much higher are obtained when compared to plant transformed using other methods (Mushegian *et al.*, 1995). This method does not transfer the DNA into the plant genome and the viral particles are generally excluded from the pollen and egg cells, hence, the recombinant DNA is not transmitted to the progeny via pollen or seeds. In addition, the viruses used in this system are poor competitors with natural viruses and over time will eliminate the introduced genes from their genome, so there is a low risk of escape and survival of the modified viruses into the environment. The limitation with this system is that the green plant matter must be processed immediately after harvest as it cannot be stored. Other types of transient expression systems include agroinfiltration which involves infiltration of a suspension of recombinant *Agrobacterium tumefaciens* into tobacco leaf tissue **so that** there is a high transfer to a very high proportion of cells, expression of transgene occurs at very high levels and magnification technology which achieve a higher

yield than the virus – vector-based expression system and *Agrobacterium-mediated* transformation (Obembe *et al.*, 2010). The agro-infiltration method has been used to either test the efficacy of an expression or to produce a small amount of proteins.

SELECTION PROCEDURES FOR THE TRANSGENIC PLANTS

It is necessary to distinguish between the transformed and non-transformed event after the transformation procedure; this is accomplished with the use of marker genes inserted along with the gene of interest. Genetic markers developed for use in plant cells can be divided into two types: selectable and screenable or reporter markers. Following the gene insertion process, plant tissues are transferred to a selective medium containing an antibiotic or herbicide depending on which selectable marker is used. Only plants expressing the selectable marker gene will survive. However, there are biosafety concerns that selectable marker genes may be toxic or allergenic when consumed. Moreover, another type of selection marker is needed in another transformation event with the transformed progeny. These selectable can be removed by either cont..

- (1) transformation with multiple T-DNA resulting in linked and/or unlinked co-integration of transgenes, unlinked transgenes are then segregated out during meiosis (Afolabiet *et al.*, 2005) (this technique is useful for seed-producing plants) or (2) marker gene excision consisting of site-specific recombination system (R/RS) (Matsunaga *et al.*, 2002). Stable expression of genes means that the plant has been stably transformed, where transgenes encoding multimeric antibody domains are permanently incorporated into the plant genome and expressed over generations. However, removal of selection – marker genes may not be necessary for the use of transgenic plants for the production of antibodies and other therapeutic agents.

PURIFICATION OF PLANTIBODIES

The commercial viability of biopharming depends on efficient methods of purification of recombinant proteins. Immunoprecipitation and chromatography are the two general methods of purification. For mAb, affinity purification on protein G or protein A column is the most useful method. The three procedures involved in antibody purification cascades are milling, extraction, and purification. Purification involves three chromatography steps, a diafiltration step and a freeze-drying step. Either a protein A or a protein G column is used to capture the antibody. The other two columns are an anion-exchange diethylamino ethyl (DEAE) column, which is used to remove impurities, and a cation exchange carboxymethyl (CM) column, which is used to remove DNA residues. The CM column also removes impurities and filters protein A ligands that may have leaked from the affinity column. The protein A

chromatography column is the key step in the purification cascade, although it is very expensive. Aqueous two-phase systems (ATPS) extraction is a technique that is well adapted to large-scale purifications and is economical and simple to use. ATPS is prepared by mixing solutions of two incompatible polymers (like dextran and polyethyleneglycol- (PEG)) or a polymer solution and a concentrated salt, for example, sodium phosphate. Liquid-liquid extraction using ATPS has been used in the separation of pharmaceuticals, including recombinant proteins, antibodies, oligopeptides, lactic acid, amino acids, enzymes, etc. Lee and Forciniti (2009) developed a new process by which human antibodies expressed in corn are isolated to high purity and yield using ATPS extraction method.

BIOSAFETY REGULATIONS

There are a lot of public concerns involved in the production and use of plant-derived antibodies; most of these fears are related to the containment of the plant during planting, growth, harvesting and disposal to ensure that these plants do not enter into the human food supply. There is also the fear that the transgenic plants biologically active products may elicit physiological responses in humans and in animals (Spoket *al.*, 2008). Unlike conventional crops plant, crop production systems for the production of recombinant proteins are grown under a closed system that is regulated by government agencies and controlled completely by the technology provider and manufacturer.

Several strategies are listed below that can be used for curtailing the effect of gene spread these include:

- i.** Prevention of pollen transfer to neighbouring crops; physical isolation distances from another field of the same species should be carried out (USDA APHIS 2002) this depends on the pollination mechanism of the plant. Plants which are self-pollinated have an enclosed flowers structure that provide good pollen containment because the risk of spread of this pollen is minimal isolation can be a relatively short distance restructure this sentence - unclear. Those plants that produce wind-dispersed pollen must be isolated for at least a mile from other fields of the same plants. The distance of separation may also depend on other strategies that are taken to limit pollen distribution such as removal of tassels and the use of male sterile in the case of corn (Gils *et al.*, 2008).
- ii.** Crops that are pollinated by insects, that produce dormant seeds, or that can cross-pollinate with related wild species growing in the area are not recommended for the production of plantibodies. The planting of crops at different periods to ensure harvesting at different periods from other crops intended for use as food and feed is also a good idea.

iii. Transgenic mitigation does not by itself prevent transgene flow from transgenic crops to non-transgenic crops or wild relatives, but 'mitigates' the effects of such gene flow. Its goal is to prevent the establishment of the transgene in volunteer populations or in populations of wild relatives if hybridization can occur (Gresselet *et al.*, 2009).

iv. Plastid transformation ensures that there is no loss of transgene as plastid can only be inherited through maternal tissue as pollen does not have chloroplast (Svabet *et al.*, 2007).

v. Genetic use restriction technologies (GURTs) which involve the use of non-germinating seeds or non-sprouting tubers/bulbs for the production of plant-made medicines is also very useful.

vi. Incorporation of site-specific recombination technologies in plant transformation allows a variety of gene excision activation control strategies. The risk of horizontal gene transfer from plants to microbes, in the case of using antibiotic resistance genes, is believed to be extremely low, as there has been no report of such a case.

CONCLUSION

The importance of plants for mankind has, hitherto, been twofold: the first being the production of free oxygen, without which evolution would not have proceeded the way it has. Mankind needs to breathe in oxygen while disseminating the unneeded carbon dioxide. Secondly, plant assimilation of carbon dioxide that mankind disseminated, giving rise to organic plant tissue, which primarily has provided nourishment for all other organisms that were not able to live on inorganic material themselves. Additionally, the production, by cont. plants, of useful organic materials like wood and numerous substances discovered because of their different effects against diseases (drugs). Currently, a third addition is in the offering; the production of antibodies and pharmaceuticals which are originally substances synthesized by humans exclusively. Antibody expression in plants has moved closer to the industrial application due to two recent research advances.

(1) High antibody expression has been obtained using transient expression and this system is expected to become predominant.

(2) Glycosylation engineering of antibodies has shown to be functional and represents an interesting tool for adapting the antibody structure to therapeutic use.

However, the problem of proteolytic degradation is still particularly troubling for two reasons; it decreases the yield of functional antibodies, and also necessitates costly steps to remove the partly degraded antibodies. In this respect, it may be necessary to develop extracellular peptidase free host plants or to engineer antibodies that possess higher resistance

to peptidases while maintaining their activity. Production of a PMP? Full name in leaves, seeds or fruits which can provide a relatively cheap, safe and “easy-to scale-up” technology is the major difference of plants to all other production systems for pharmaceuticals (e.g. mammalian cells, bacteria, yeasts, fungi). From an economical point of view, plants might one day surpass the other production systems, if the yields of desired recombinant protein can be further increased. But, the commercial success of the plant production system probably, is not as much determined by solving the technological hurdles as it is by biosafety issues and the development of public acceptance. Only if all of these problems can be overcome, It is possible, in non-distant future, that therapeutic agents can be produced on a scale that meets worldwide demands, and also those who need them most can afford them if these challenges are met.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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