Chapter

Development of an Oral Vaccine for the Control of Cysticercosis

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Abstract

Parasitic diseases fecally transmitted, such taeniasis/cysticercosis *Taenia solium* binomial, represent a health problem whose incidence continues due to the prevalence of inadequate sanitary conditions, particularly in developing countries. When the larval stage of the parasite is established in the central nervous system causes neurocysticercosis a disease than can severely affect human health. It can also affect pigs causing cysticercosis causing economic losses. Since pigs are obligatory intermediate hosts, they have been considered as the targets for vaccination to interrupt the transmission of the parasitosis and eventually reduce the disease. Progress has been made in the development of vaccines for the prevention of porcine cysticercosis. In our research group, three peptides have been identified that, expressed synthetically (S3Pvac) or recombinantly (S3Pvac-phage), reduced the amount of cysticerci by 98.7% and 87%, respectively, in pigs exposed to natural conditions of infection. Considering that cysticercosis is orally acquired, it seems feasible to develop an edible vaccine, which could be administered by the pig farmers, simplifying the logistical difficulties of its application, reducing costs, and facilitating the implementation of vaccination programs. This chapter describes the most important advances towards the development of an oral vaccine against porcine cysticercosis.

Keywords: cysticercosis, *T. solium*, oral vaccine, transgenic plant, *Carica papaya*

1. Introduction

Taenia solium taeniasis/cysticercosis is a parasitic zoonosis that significantly affect economic and public health. Neurocysticercosis (NCC) is a most severe form of the disease caused by the establishment of the larval stage (cysticerci) of *Taenia solium* in the central nervous system (CNS). In 2010, the World Health Organization declared it one of the leading neglected diseases and aims to develop strategies for its eradication and prevention [1].

Between control measures it has been explored the improvement of health education, sanitary conditions, standards of meat inspection and the rearing of pigs. It has also been explored the impact of massive or individual treatment of taeniasis and the treatment and/or vaccination of pigs, all of them with promising results [2–4]. Vaccination of pigs would imply an unlikely immediate and potent effect

upon the number of tapeworm-carriers in rural communities, interrupting the parasite's life cycle and eventually reduce human neurocysticercosis. Developing an effective vaccine against *T. solium* pig cysticercosis is also being pursued by different research groups with promising results [5, 6].

In our group, an anti-cysticercosis vaccine named S3Pvac based on three peptides expressed was developed. The vaccine synthetically (S3Pvac) or recombinant (S3Pvac-phage) produced, reduced the number of cysticerci by 98.7% and 87% [7, 8] in randomized field trials, respectively. The recombinant vaccine was subsequently used in a control program applied in the State of Guerrero, confirming its usefulness. Indeed, S3Pvac-phage significantly reduce the prevalence of porcine cysticercosis from 7 to 0.5% and 3.6 to 0.3% estimated by tongue examination or ultrasound, respectively [3]. In the course of this control program, we were able to evaluate the difficulties involved in using an injectable vaccine. Pigs are produced free rurally reared, thus the application of an injectable vaccine requires their capture and subjection, a laborious procedure that increases the costs of vaccination and limits its massive application. Considering that cysticercosis is orally acquired, it seems feasible to develop an oral vaccine [9], which could be administered by the pig breeder, simplifying the logistical difficulties of its administration, reducing costs and facilitating the implementation of vaccination control programs.

For the design of an oral vaccine the use of plants is increasingly recognized as valuable platform. Plants offer the production of antigens at low costs, circumventing costly purification procedures. Plants also offer a natural way of antigen encapsulation preventing its degradation by the detrimental environment to which an oral intake vaccine is exposed such us antigen degradation by low pH, mucosal enzymes [10, 11] and the use of cell cultures will avoid non-desirable environmental effects due to the release of transgenic plants into the environment.

Moreover, plants also frequently include components with adjuvant properties like saponins that may increase the immunogenicity of the vaccine [12]. Considering this, the recombinant peptides KETc7, KETc1.6His, and KETc12.6His were expressed in transgenic clones of papaya embryogenic calli [13]. The three clones together constitute the oral S3Pvac-papaya vaccine candidate. Papaya was selected because the high efficiency of transformation and its own antiparasitic properties [14].

This third version of the vaccine has been shown to be immunogenic in mice and pigs and is being produced in suspended culture systems to massively produced this oral version of the vaccine that must be evaluated on the field against pig cysticercosis.

2. Parasite and oral immunity

Oral vaccination is an interest route to prevent infections caused by orally acquired pathogens overcoming the limitations of current injection-based vaccines in providing front-line protection against pathogen invasion and dissemination [15]. It offers a painless, safe and low-cost route that does not require trained personnel. Moreover, this route can elicit mucosal and systemic immunity. Vaccine antigen can be recognized and translocated by M cells, which act as sentinels and enter directly into the Peyer's patches. Then antigens can be transported to the intestinal mesenteric lymph nodes, stimulating the host's systemic and mucosal immune response resulting in the production of IgA and IgG antibodies with the ability to neutralize of invading pathogens before they are able to cause a widespread infection. Oral vaccination can also trigger an effective cellular immunity. However, the development of oral vaccines is a major challenge due to an inefficient

Pathogen	Plant	Protein	Route	Species	Reference
Rabies virus	Tomato	GP	ND	ND	[16]
Hemorrhagic Virus	Potato	VP60	Sc, Im	Rabbit	[17]
Rotavirus A	Potato	VP6	Ip	Mice	[18]
Foot and mouth virus	Arabidopsis	VP1	Ip	Mice	[19]
Foot and mouth virus	Potato	VP1	Ip	Mice	[20]
Foot and mouth virus	Alfalfa	Peptide VP1 β-glucuronidase	Ip	Mice*	[21]
Gastroenteritis virus	Arabidopsis	GP- S	Im	Mice	[22]
Gastroenteritis virus	Potato	GP- S	Oral	Mice	[23]
Gastroenteritis virus	Tobacco	GP- S	Ip	Pig	[24]
Gastroenteritis virus	Seeds of corn	GP- S	Oral	Pig*	[25, 26]
S3Pvac-papaya	Embryogenic Transgenic clones	KETc1, KETc12 and KETc7 peptides	Sc	Mice	[13]
S3Pvac-papaya	Embryogenic Transgenic clones	KETc1, KETc12 and KETc7 peptides	Oral	Pig	[27]

**Protection against infection.*
Sc: subcutaneous; Im: intramuscular; Ip: intraperitoneal, ND: Not determined; GP: Glycoprotein.

Table 1.
Expression of antigens aimed at veterinary vaccine development.

transport to reach M cells and the possibility to induce local and systemic immune tolerance. Considering that plant-based vaccines usually expressed low content of antigen it seems feasible to avoid oral tolerance using the proper dose and vaccine schedule. It remains to be elucidated if plants-derived vaccines could overcome mucosal tolerance when administered to human beings.

Table 1 shows various plants that have been used to express antigens from different pathogens to be evaluated as edible vaccines. Tobacco has been used as an experimental model of transformation and expression. However, the use of other species such as tomatoes, lettuce, potatoes, corn, soybeans, alfalfa, Arabidopsis, papaya and carrots has been expanded [11, 28–33]. In some of these plants, the expressed recombinant antigen has shown efficacy when evaluated in experimental models or directly in the naturally affected host. Recombinant antigens have been reported to induce an immune response with the production of IgG, IgM or IgA antibodies, regardless of the route of administration [31].

3. Transgenic plant platform

Many different advantages of expression of recombinant proteins in transgenic plants for vaccine production can be mentioned over other commonly expression systems, such as bacteria, yeasts and baculoviruses. Plants can be constitutively or tissue-specific

expressed in single or multiple transgenes, antigens can be stable in seeds without the requirement of refrigeration, no purification nor cold chain for preservation.

Transgenic plants can also be used as bioreactors to produce high amounts of the recombinant protein of interest [34, 35]. They can also be produced as *in vitro* tissue culture, cell suspensions, hairy roots, moss protonema, microalgae and whole plants. There are many experimental plant-made veterinary vaccines produced in seeds, fruits, and leaves, that can be orally delivered as part of the animal feed, thus offering great convenience and economy in immunizing large populations of animals on farms [35]. The expression of antigens for the production of vaccines in transgenic plants is considered a safe and effective immunization system, which can avoid some of the difficulties associated with traditional vaccination methods, as well as a reduction in the costs of production, distribution and conservation.

One nice study of veterinary interest is the expression of the glycoprotein S of the porcine gastroenteritis virus in corn seeds for the production of an oral vaccine, which has also the ability to induce protection, through colostrum, in piglets [25, 26].

3.1 *Carica papaya* L.

Classification of *Carica papaya L.*
Family: *Caricaceae.*
Gender: *Carica.*
Species: *C. papaya L.*
Morphological type: Arboreal.
Climate: Equatorial tropical.

Carica papaya is a species of pantropical plant that grow in tropical regions of the Americas from Mexico to Argentina, Africa and Asia. The main importers are: United States, Japan, Hong Kong and the European Union. *Carica papaya* is known by different common names such as capaidso, naimi, nampucha, pucha, fruit bomb, milky, mamao, pawpaw. Papaya is an arborescent, semi-perennial plant that grows in areas with an average rainfall of 1800 mm per year and an average annual temperature of 20–22°C, a large number of varieties have been developed. Papaya fruiting occurs 10 to 12 months after transplantation, is maintained for ten years, and female, male or hermaphrodite [36] trees can be obtained. Papaya is a fruit known for its nutritional benefits and medicinal properties. Main papaya components and their reported properties are shown in **Table 2**.

3.2 *Carica papaya* as a cestode vaccine

Papaya is an alternative system for the exploration of tropical tree genomes, containing a genome of 372 megabase (Mb), of diploid inheritance with 9 pairs of chromosomes and presents the smallest gene number, 24,746 genes [37]. Papaya exhibits some properties of possible advantages to be used as a platform to express *T. solium* vaccine antigens. Papaya components have antiparasitic properties per se [14]. Cells can be easily transformed by bioballistics and *in vitro* propagated and regenerated [38].

Among many papaya components, the papain contained in the latex has been widely evaluated in its ability to damage the cuticle of intestinal parasites by proteolytic digestion. **Table 3** shows some reports on the characterization and evaluation of antiparasitic activity of papaya against *Trichostrongylus colubrormis, Heligmosomoides polygyrus, Trichuris muris, Protospirura muricola* [39, 44–49] *Rodentolepis microstoma* [39], *Hymenolepis diminuta and microstoma* [40].

Composition		**Properties**	
Nutrimental	**Enzymatic**	**Fruit**	**Seed**
Carbohydrates	Papain	Digestive	Oxytocic
Sugars	Chymopapain	Healing	Vermifuge
Food fiber	Caricain	Strengthens Immunity	Spermatocide
Fat	Glycyl endopeptidase	Antibacterial	Emmenagogue
Proteins	**Lesser amounts**	Contraceptive	
Retinol (vit. A)	Class II chitinase	Analgesic	
β-carotene	Class III chitinase	Antibiotic	
Thiamine (vit. B_1)	Serin protease inhibitor	Stimulation of pancreatic juices	
Riboflavin (vit. B_2)	Glutamyl cyclotransferase	Hypotensive	
Niacin (vit. B_3)	Beta-1,3-glucanase	Febrifuge	
Pantothenic acid (vit. B_5)	Cystatin	Anti-inflammatory	
Folic acid (vit. B_9)	Acetogenins*	Anti-helminthic	
Vitamin C	Carpasemine		
Vitamin E			
Vitamin K			

**Antiparasitic activity. Calcium, iron, magnesium, manganese, phosphorus, potassium, sodium and Zn are also included in papaya.*

Table 2.
Papaya components and medicinal properties reported.

Evaluation	**Component**	**Treatment**	**Results**	**Reference**
In vitro				
Rodentolepis microstoma	Papain (Sigma) Raw latex (Sigma)	25 mM	Disrupt the surface of the cuticle	[39]
Hymenolepis diminuta microstoma	Latex supernatant Commercial papain	Not available	Damage to the strobile Reduced motility and subsequent death of the parasite	[40]
Anoplocephala perfoliata	Cysteine-proteinases Latex supernatant	300 mM	Reduced motility and induced death of the parasite	[41]
Hymenolepis diminuta	Papaya latex supernatant	2.4 μmol	Affected worm growth	[42]
In vivo **(mouse)**				
Hymenolepis microstoma	Latex supernatant	240 nm for 6 days	Minimal and temporary efficacy	[43]

Table 3.
Antiparasitic activity of papaya against some cestodes.

Anoplocephala perfoliate [41] *Hymenolepis diminuta* [42] *Hymenolepis microstoma* [43] without causing side effects to the host [50, 51].

3.3 S3Pvac-papaya anticysticercosis vaccine

For the development of the S3Pvac-papaya cysticercosis vaccine, three genetic constructions were used for the expression of recombinant peptides, KETc1.6His, KETc12.6His and KETc7 [13]. **Figure 1** summarize the methodology employed for the production of S3Pvac-papaya vaccine.

3.4 Protective properties of S3Pvac papaya against cysticercosis

The S3Pvac vaccine expressed in embryogenic papaya clones has demonstrated high protective capacity against experimental murine and *T. pisiformis* cysticercosis orally administered. **Table 4** shows the protective effect induced by oral immunization in mice at a dose range of 0.1 to 1 μg of soluble extract, whilst a higher dose lowered the percent of protection. In addition, different vaccine formulations also reduced the expected parasite load. On the other hand, the oral vaccine significantly reduced the number of infected rabbits and the percentage of cysticercus-free animals (83%), 21 days after the infection. The S3Pvac-papaya vaccine has not yet been evaluated in pigs, however, its immunogenic response in mice and pigs [27, 52], and its protective capacity in different models exhibit its potential to exert a protective response against naturally acquired porcine cysticercosis.

We previously reported non-specific protection that was induced by the wild type soluble extract [52, 54] has been attributed to the antiparasitic properties described to papaya itself mentioned above.

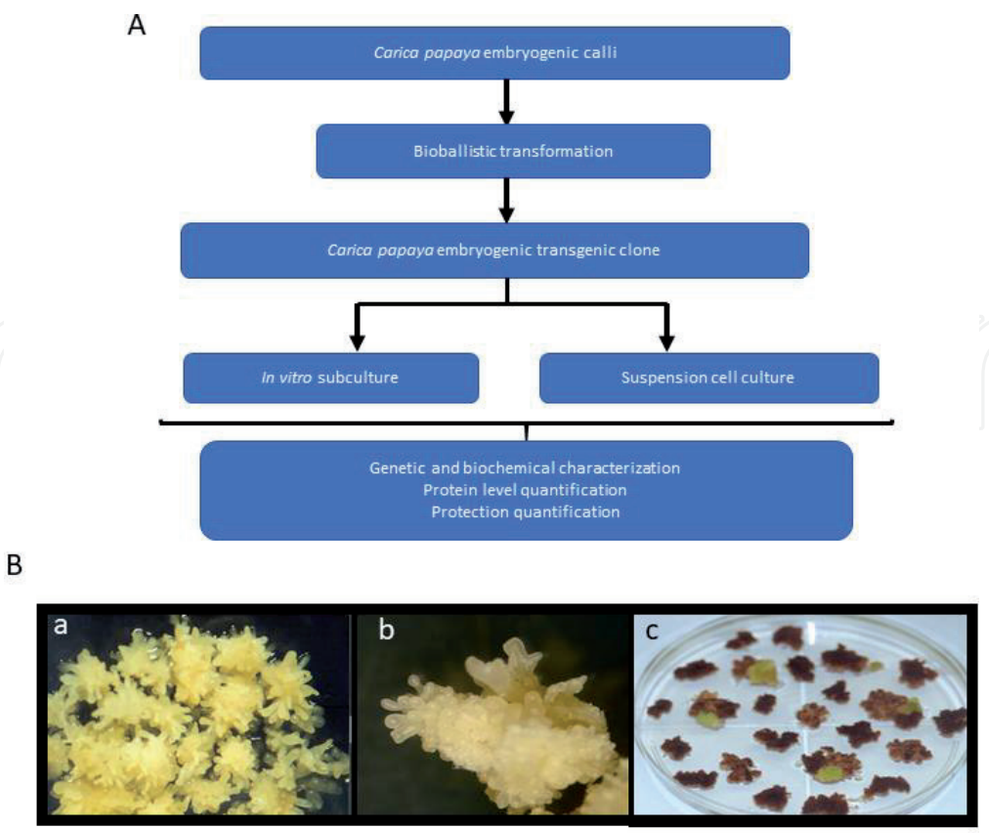

Figure 1.
(A) Production of transgenic embryogenic papaya clones by a bioballistic method (B) Embryogenic papaya callus: a) Induction of embryogenic callus; b) Embryos in globular stage for transformation; c) Selection of transformed clones in selective medium.

Experimental model	Immunized with:	Mean ± SD (% Protection)[†]	Immune response	Reference
				[27]
	Saline	27.2 ± 14.2		
	S3Pvac papaya saline (μg/dose)			
Murine *T. crassiceps* cysticercosis	0.1	12.3 ± 3.4* (55)	CD4, CD8 proliferation	
	1	10.8 ± 2.5* (60)	CD4, CD8 proliferation	
	10	9.2 ± 1.0* (66)	Specific IgG Abs; CD4 and CD8 proliferation	
	100	41.4 ± 62 (0)		[52]
	Saline	18.6 ± 17.3* (86.8)		
	Corn starch[§]	29.8 ± 31.4 (41.4)		[27]
	Soy oil	10.3 ± 2.2* (75)		
	Canola oil	11.5 ± 8.6* (84)		
Rabbit experimental *T. pisiformis*	Saline	4.33 ± 4.01		[53]
	S3Pvac papaya[£]	0.25 ± 0.62* (94)		

[†]Mean ± standard deviation of the number of cysticerci recovered in each group.
[§]Mice were fed with S3Pvac-papaya soluble extract (1 μg of total protein) into different vehicles.
[£]Rabbits received a suspension of 20 mg of each embryogenic transgenic papaya clone expressing KETc1, KETc12 and KETc7 in a gelatin capsule.
**Protection statistically significant ($P < 0.05$).*

Table 4.
Protective capacity induced by oral S3Pvac-papaya vaccine against experimental.

3.5 Biotechnological approach for the production of papaya anti-cysticercosis vaccine

Plant biotechnology is a rapidly evolving area with major impact in the production of molecules with high pharmaceutical value. *In vitro* culture techniques offer central advantages in the manufacture of desired chemicals for human health. The benefits include a systematic supply of compounds under optimized controlled conditions, independence of weather, soil, disease, and socio-political problems; discovery of new compounds, bio-transformation systems and better adaptation to market changes. In an inclusive context, *in vitro* systems will give a better understanding of plant biochemistry and physiology, as well as some basic aspects of plant differentiation.

Plant biotechnology involves relevant procedures in the manufacture of oral vaccines enabling the production of higher amounts of active biomasses from transgenic plants, by means of massive propagation of cells, tissues and organs [12, 55]. Among others, these procedures include the growth of callus (aggregates of undifferentiated cells growing in solid media), suspension cultures (individualized undifferentiated cells growing in liquid media); as well as embryo cultures that could be grown in solid or in liquid nutrient media.

The three callus lines expressing KETc1, KETc7 and KETc12 peptides were generated, and further efforts to optimize the massive growth of the corresponding callus and suspension cultures, were conducted. These *in vitro* systems constitute adequate platforms for the massive production of papaya anti-cysticercosis vaccine in the near future.

3.5.1 Callus cultures

In the establishment and optimization of callus cell lines, *Carica papaya* L. (KETc7) embryogenic calli were used to obtain friable undifferentiated cells. Calli were placed in solid culture medium with 30 g/L sucrose, 3 g/L^{-}polivinilpilorridone and 1.5 g/L^{-}of phytagel. The nutrient media MS [56] and B5 [57] were evaluated; and the presence of the phytoregulators 2,4-dichlorophenoxyacetic acid (2,4-D) at 1.0, 2.0 and 3.0 mg/L^{-}combined with kinetin (KN) at 1.0, 2.0 and 3.0 mg/L, was also tested. The cultures were maintained at 25°C and subjected to constant light as well as dark conditions. The best results were obtained for callus growing in B5 medium with 2 mgL^{-1} of 2,4-D combined with 2 mgL^{-1} KN in dark conditions (**Figure 2**). In these conditions after two subcultures non-phenolized calluses were developed, and after several subcultures the friable callus KETc7 cell line, was established.

3.5.2 Cell suspension cultures

Ten grams fresh weight (FW) of the friable callus line KETc7 were inoculated in 250 ml Erlenmeyer baffled flasks containing 100 ml nutrient medium without phytagel, and placed for 30 days on a rotary shaker at 100 rpm, 25°C and dark conditions. To disaggregate cell clusters and increase oxygen transfer, baffled flasks were used (**Figure 3**).

The cultures were sub-cultured in fresh medium every 15 days, and the best results were observed when using B5 nutrient medium, cultivated in darkness, and producing uniform suspended cultures without phenolization (**Figure 4**).

Cell viability was maintained at 95% until 45 days in culture, as confirmed by the fluorescein diacetate (FDA) method (**Figure 5**) [58].

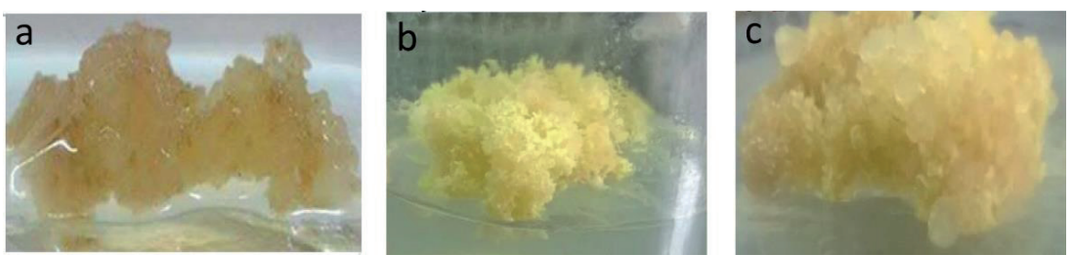

Figure 2.
Optimization of C. papaya KETc7 callus cell line under different growth conditions. (a) Photoperiod, (b) constant light, (c) darkness.

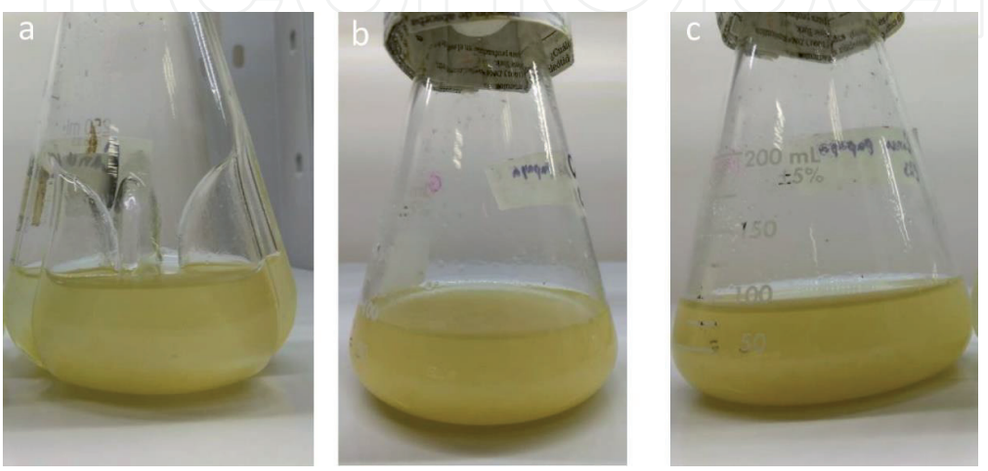

Figure 3.
Establishment of C. papaya KETc7 cell suspension line. (a) Culture in baffle flasks at 15 days (b) culture in Erlenmeyer flasks at 15 days (c) culture in Erlenmeyer flasks at 30 days.

Once the friable uniform cell suspension cell line was established, it became possible to evaluate the growth kinetics of the culture during 45 days by collecting samples every 3 days, and determining the following growth parameters: fresh weight, dry weight, cell viability, pH and carbohydrate consumption [59].

The results showed that the KETc7 suspension cell line grew very well, reaching a doubling time of 6.9 days and a specific growth rate (μ) of 0.10 d^{-1}. The maximum biomass value was 14.36 gPS L^{-1} obtained at 15 days in culture.

3.5.3 Embryo suspension cultures

The KETc7 embryos callus cell line generated in solid B5 medium without phytoregulators was used to establish embryo suspension cultures. An inoculum of 10% was added into 250 ml Erlenmeyer flasks containing 100 ml of liquid B5 medium (**Figure 6**). The flasks were kept on an orbital shaker at a stirring speed of 115 rpm, under constant light conditions (24 μmol.m^{-2}. S^{-1}) and 25°C. After 15 days, the biomass was sub cultivated in the same conditions described above, and the culture was propagated.

3.5.4 Cell suspension cultures in bioreactors

To scale-up the *C. papaya* suspension cultures 2 L airlift bioreactors were employed, with the following geometric design: height (52 cm), diameter (7 cm), draft tube height (27 cm), diameter of inner draft tube (2.7 cm), and bottom clearance (2.0 cm) [60]. The air was sprayed at the bottom of the draft, generating an internal loop in which the upcomer is in the draft, and the downcomer in the ring.

Figure 4.
Growth of C. papaya KETc7 cell suspension line at 15 days in a rotary shaker at 100 rpm, 25°C, in the dark.

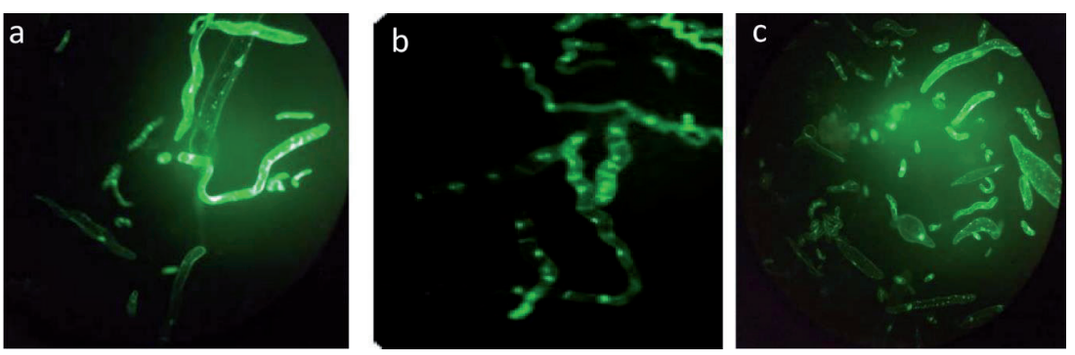

Figure 5.
Cell viability of C. papaya KETc7 cell suspension line by the fluorescein diacetate (FDA) method at day a) 0, b) 7, and c) 15, in an Epifluorescence Microscope Nikon Eclipse E400 (40X).

The bioreactor was sterilized and then filled with autoclaved B5 medium (1.8 L) supplemented with 30 g/L sucrose, 2,4-D and KN (2 mg/L each). Fifteen-days-old *C. papaya* KETc7 cell suspension line was used to obtain an inoculum of 10% (v/v) FW. The culture in bioreactor was incubated at 25 ± 2°C under continuous light (white light flux density of 50 μmol/m^2/s) for 30 days. The bioreactor was operated in a batch mode at 0.1 vvm for 15 days and subsequently at 0.8 vvm, until the end of the culture period. Under these conditions, an adequate mixing of the cell suspension was obtained. Antifoam (Dow Corning FG-10) was applied as required, by injection of 0.5 mL (0.1% v/v). The culture was sampled every three days and the concentration of biomass, pH and sugars, was determined. Results show that the K ETc7 cell suspension culture was able to grow uniformly and that the exponential growth phase was reached from days 6 to 12, followed by a stationary phase. The maximum biomass was of 18.6 ± 0.7 g/L DW (**Figure 7**).

3.5.5 Embryo suspension cultures in bioreactors

Growth of *C. papaya* KETc7 embryo suspension line was scaled-up in the 2 L airlift bioreactors described before. Two weeks old embryo suspension line was used to obtain an inoculum of 10% (v/v) FW. The culture in bioreactor was incubated at 25 ± 2°C under continuous light (white light flux density of 50 μmol/m^2/s)

Figure 6.
C. papaya KETc7 embryo suspension line grown in B5 medium at 100 rpm, 25°C, and constant light (24 μmol. m^{-2}. S^{-1}).

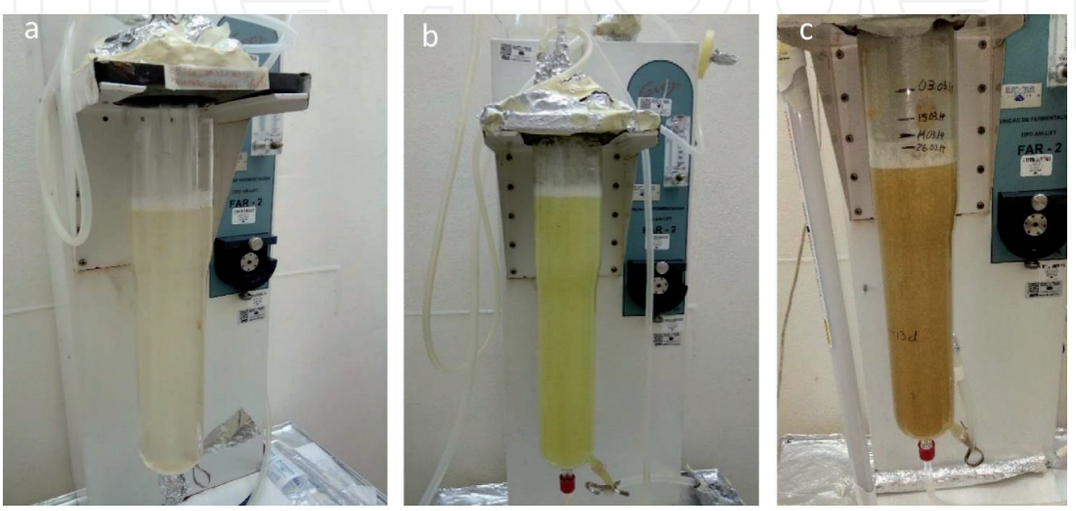

Figure 7.
C. papaya KETc7 cell suspension line growing in airlift bioreactor: (a) day 0, (b) day 15, (c) day 30.

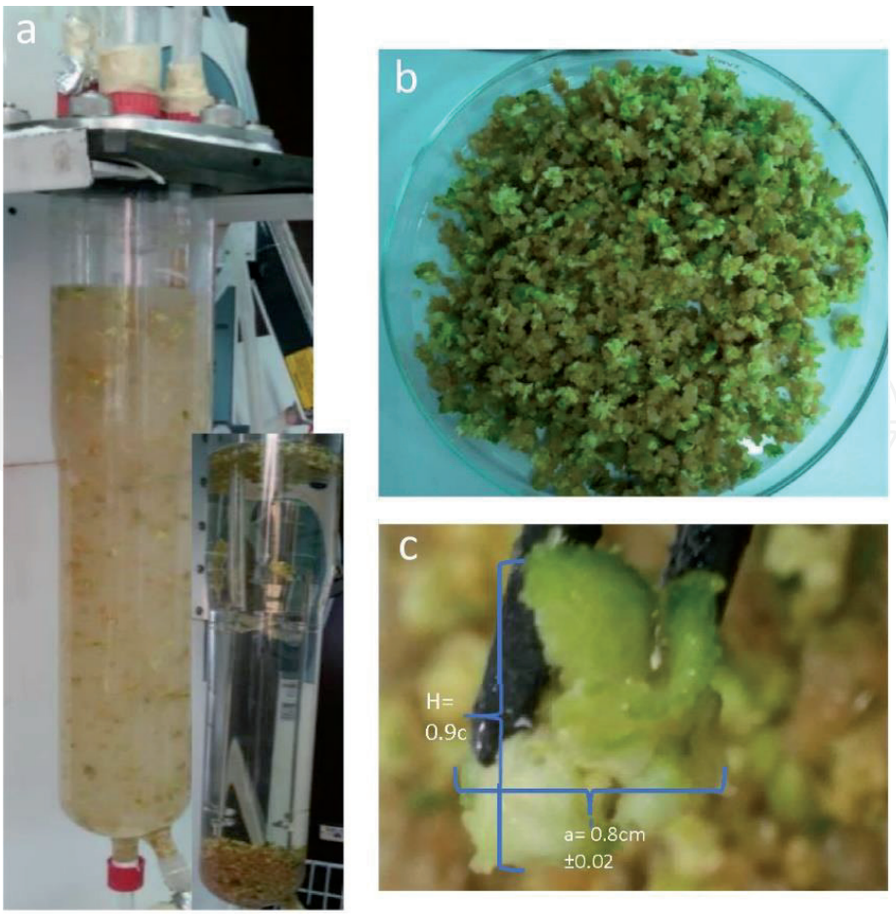

Figure 8.
C. papaya KETc7 embryo suspension line growing in airlift bioreactor (a) embryo culture in airlift bioreactor at 30 days, (b) harvested embryos after 30 days in culture, (c) embryos observed in stereoscopic microscope (10×).

for 30 days. The bioreactor was operated in a batch mode at 0.1 vvm for 15 days and subsequently at 0.8 vvm until the end of the culture period. Embryo culture of the line KETc7in bioreactor batch type process, showed uniform growth. A maximum biomass of 30 g/L DW was obtained, which represents 4 times more respect to the initial inoculum and the number of generated embryos was of 279 (**Figure 8**).

4. Conclusions

This review addresses oral vaccination as a feasible approach to prevent parasitic diseases. Since most anti-parasitic vaccines currently available are parenterally administered, their use involves high production and logistic costs and become inaccessible for underdeveloped countries. To cope with this issue, the use of papaya transgenic clones is herein proposed to develop an anti-cysticercosis oral vaccine and to assay its effectiveness against other parasitic infections of veterinary and/or public health interest. The use of biotechnological tools by escalation of suspension cultures would allow us to produce a vaccine in sufficient, controlled amounts for its direct application, reducing the use of antibiotics, and therefore the risk of bacterial resistance.

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Conflict of interest

The authors declare no conflict of interest.

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