Working together to eliminate cyanide poisoning, konzo, tropical ataxic neuropathy (TAN) and neurolathyrism

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Howard Bradbury[+] - Hon. Chairman and founder (1926-2016)
Fernand Lambein[+]- Hon. Coordinator (1938-2020)

EDITORIAL

This is our second edition of this year 2020. It would not have been easy for us to gather all these materials without your collaboration. I would therefore like to take this opportunity to thank all the members who have sent us their articles. We continue to solicit your contributions to research and news in future editions of our bi-annual newsletter, CCDN News.

Our lives have been disrupted this year by the pandemic of COVID 19, the socio-economic consequences of which have been devastating. The spread of this disease has severely limited our daily movements and contacts. Governments have had to impose restrictions on their fellow citizens, sometimes confining them to their homes, and imposing obligatory barriers. The appearance of new cases of konzo and neurolathyrism is to be feared because the food system has been hit hard by the reduced exchange between communities. Vulnerable populations in remote areas where cases of these two diseases have been reported have been less able to diversify their diet and have fallen into a monotonous dependence on cassava and the lathyrus pea, respectively.

On top of this, the year 2020 has been particularly painful for our CCDN network as we have lost two leaders, eminent grey eminences, and great researchers in the field of konzo and neurolathryrism. Professor Fernand Lambein passed away on the 4th March 2020 as a result of an illness that he had been fighting for several years. He was professor and head of the Laboratory of Physiological Chemistry, Department of Biochemistry, Faculty of Medicine at Ghent University Belgium. After his retirement in 2003, he joined IPBO (Institute of Plant Biotechnology Outreach) established by Prof Marc Van Montagu (The World Food Prize Laureate 2013) and continued Lathyrus research. He coordinated international collaborations with Bangladesh,

Canada, Ethiopia and India on Lathyrus and lathyrism. From 2008 to March 2020, he was the coordinator of our CCDN network. He was the author of more than a hundred publications in the field of lathyrus and konzo (see Web of Science Core Collection: https://app.webofknowledge.com/author/record/285324 and Library of Ghent University: https://biblio.ugent.be/person/F338AECC-F0ED-11E1-A9DE-61C894A0A6B4).

Professor Jean Pierre Banea passed away on the 13th September 2020 from a cardiovascular accident. He had been the director of the National Centre for Planning of Human Nutrition (CEPLANUT), which became the national nutrition programme (PRONANUT), of the Democratic Republic of the Congo (DRC), over a long period. He was a leader in konzo research and prevention in the DRC. In 1997, he obtained his PhD at the University of Uppsala on the topic of dietary exposure to cyanogens from cassava and continued with a prolific research output on konzo and cassava. He also succeeded in making konzo a priority in the strategic nutrition plan and the health development plan of the DRC. From the early 2000s, he taught at the Kinshasa Institut Supérieur de Techniques Médicales/ Dietetics and Nutrition Section and at the University of Kinshasa /Faculty of Medicine/School of Public Health. He was a member of the editorial committee of our biannual newsletter CCDN News and was the author of many publications on konzo (see https://scholar.google.be/scholar?hl=nl&as_sdt=0%2C5&q=banea+mayambu+jp&oq=Banea+Mayambu).

They are gone but are still with us. They played a great role in my life as a student and as a professional. I thank them deeply from my heart and salute their memories.

Delphin DIASOLUA NGUDI

ARTICLES

Biochemical and toxicological aspects of β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP): Importance of oxidative stress and social stress on the pathogenesis of neurolathyrism

We dedicate this article to the late Prof. Fernand Lambein who has passed away on 4 March, 2020 in Gent.

Here, we review our work with Prof. Fernand Lambein, during more than 40 years, of the biochemical and toxicological aspects of neurolathyrism, which is caused by grass pea and its toxic component β-ODAP.

Grass pea (*Lathyrus sativus*) is an important legume crop grown mainly in South Asia and Sub-Saharan Africa, however, its use is limited because of the presence of an endogenous neurotoxic non-protein amino acid β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP). Long-term consumption of grass pea and β-ODAP is linked to neurolathyrism (NL), which is an irreversible motor dysfunction.

Neurolathyrism and Konzo (caused by *Manihot esculenta*, or cassava) are largely forgotten diseases, but patients are still found in under-developed countries. NL is characterized by spastic paraparesis observed mostly in the lower extremities along with other symptoms such as increased muscle tone, spasticity, Babinski's sign, and spastic gait suggesting that it is a disease of the corticospinal tract composed of lower as well as upper motor neurons (MNs) without sensory or cognitive dysfunctions. NL causes profound socio-economic disadvantages to patients throughout their lives. It is caused by a toxic amino acid β-ODAP, which is contained in grass pea at about 0.5 % concentration (ranging from 0.07 to 1.5 %) of dry weight. The most recent outbreak of NL occurred in Ethiopia in the late 1990s and there were thousands of victims. However, patients were also seen in India, Bangladesh, Algeria, Afghanistan, some countries of Europe and China where grass pea is cultivated or grows naturally. Grass pea has been a life-saving plant especially for low-income people as the legume is rich in protein (up to 29 %) with good taste. A drawback of this legume other than containing β-ODAP is that the sulfur-amino acids methionine and cysteine/cystine are the limiting amino acids contrary to a rich content of lysine. Previous outbreaks of NL coincided with periods of prolonged food shortage (famine), when grass pea was the only food available. The highest incidences of NL at 66 % were observed at the German concentration camp in the Ukraine during WWII and during the famine in 1971-75 in Bangladesh. In these cases of high incidence, substantial periods of continuous intake of grass pea for 2 to 3 months without taking other cereals (and/or animal proteins), which supply the limiting amino acids, was the background to the outbreaks. War, imprisonment and famine are specified situations to consider.

In the 1980s, our team joined Prof. Lambein on the biochemical study of non-protein β-substituted alanines, including isoxazolinone derivatives such as β-(isoxazolin-5-on-2-yl)-L-alanine (BIA) in *Lathyrus* plants. BIA had been isolated as a prominent metabolite during the seedling stage of grass pea and of some other closely related legumes by Prof. Lambein *et al*. Using radioactive labeled compounds, BIA was confirmed to be the biosynthetic precursor of the neurotoxin β-ODAP in grass pea, and BIA was proposed to be converted to

the short-lived intermediate α,β-diaminopropionic acid (DAPA), which was subsequently oxalylated by oxalyl-coenzyme A to form β-ODAP. Production of BIA is essential for β-ODAP synthesis and appears linked to the normal metabolic activities of cysteine synthase (CSase). In cysteine biosynthesis, CSase catalyzes cysteine formation from *O*-acetyl-L-serine (OAS) and hydrogen sulfide. As thus, it was also proven that BIA is enzymatically synthesized *in vitro* from OAS and the free isoxazolin-5-one ring, using CSase purified from grass pea seedlings[1]. Furthermore the enzymatic breakdown of BIA with formation of DAPA was catalyzed by an enzyme partially purified from grass pea seedlings[2]. The enzyme activity was dependent upon Ferrous-ions (Fe^{2+}), but the addition of Mg^{2+} had no effect on this enzymatic breakdown reaction. The presence of Fe^{2+} seems to have a great impact on this enzymatic activity because of no catalyzing formation of DAPA by enzyme preparations from sweet pea (*L. odoratus*) seedlings, in which β-ODAP was not found. This is the first evidence for the presence in *Lathyrus* plants of an enzyme that breaks down BIA with formation of DAPA and confirms the pathway to β-ODAP from BIA *via* DAPA proposed earlier by Prof. Lambein *et al*. This is probably the key enzyme in the biosynthesis of the *Lathyrus* neurotoxin that is responsible for the human neurodegenerative disease lathyrism, because in the pathway leading to β-ODAP, the earlier precursor BIA is also present in the garden pea (*Pisum sativum*) and in lentil (*Lens culinaris*), where no β-ODAP was found. Sequencing the enzyme would be an important step closer to the genetic transformation of *Lathyrus* plants in order to make it free of toxin and be also important for improvement of the nutritional status of people in Asia and Africa who survive on this legume in periods of drought.

In the 1990s, we extended our interest of the neuro-pathological study of NL based on our studies on the glutamatergic neuronal system, a major excitatory neuronal system, which is related to various neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS). Through our *in vitro* studies, the following discoveries were made. Commercially available β-ODAP showed neurotoxic activity through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamatergic receptor (AMPA-R) in rat primary cortical neurons, extending the findings of Dr. P. B. Nunn and other researchers done in the 1980s. Using primary motor neurons (MNs) from rat spinal cord, however, AMPA-R antagonists did not completely prevent the cell death caused by 300 μM β-ODAP, leaving about 30% of them unaffected, which needed further explanation[3]. Ca^{2+} imaging analysis of MN revealed that β-ODAP induced slow and prolonged intracellular Ca^{2+} ($[Ca^{2+}]i$) elevation in primary MN, and it correlated well with the resultant MN death. Analyses by this system clarified that the effect of β-ODAP on $[Ca^{2+}]i$ was different from that induced by (*S*)-AMPA (a typical AMPA-R agonist) in several aspects. Most of the $[Ca^{2+}]i$ increased by β-ODAP was independent of the concomitant Na^+ influx or depolarization as it was not abolished by tetrodotoxin (TTX), whereas that of (*S*)-AMPA was completely inhibited by TTX. Thus, β-ODAP acted through some different pathways from those of (*S*)-AMPA to increase $[Ca^{2+}]i$. One of the targets we found were the transient receptor potential channels (TRP)-M2 and (TRP)-M7[3]. As these TRP-M channels are sensitive to reactive oxygen species to enhance $[Ca^{2+}]i$ by increasing the Ca^{2+} influx, the toxic pathway of β-ODAP on MN was thus proved not only mediated *via* AMPA-R-triggered, voltage-dependent Ca^{2+} channel but *via* other pathway(s) related to oxidative stress. As sulfur-amino acids (methionine and cysteine) were quite low in grass pea, and as these amino acids are important in maintaining glutathione levels, we hypothesized that the deficiency in sulfur amino acids would have potentiated the toxicity of β-ODAP to MN, and the situation correlates with the monotonous intake of grass pea that was the case in the concentration camps and in famine conditions. Using a MN cell line NSC-34, the toxicity of β-ODAP on the neurons was potentiated by depriving the assay medium of methionine and cysteine and was reversed by supplementation with these amino acids. These results propose a new insight into the mechanism of NL in which β-ODAP induces the MN-specific death when glutathione levels are reduced[4].

We developed a rat model to study the neurological/pathological mode of action of β-ODAP. When newborn rats were treated with β-ODAP parenterally (subcutaneously, s.c.), they were quite responsive to the neurotoxin showing seizure; whereas five to seven days after birth, they are not responsive to the toxin at all at doses of 200 to 400 mg/kg, reflecting that their blood-brain/spinal cord barrier was already working. We first examined the distribution of β-ODAP in the central nervous system of newborn rats[5]. The parenteral β-ODAP distributed mainly to spinal cords (higher level in the lumbar-sacral portions than in the thoracic-cervical portions) with a trace amount found in medulla oblongata and pons/midbrain, but none was detectable in cerebral cortex/striatum/thalamus and cerebellum. Thus, in the newborn animals, β-ODAP distributed at the highest level into the lower spinal cord segments. Then, we tried to establish a NL model of the rat. Treating newborns with 200 mg/kg of β-ODAP s.c. continuously for five to seven days after birth produced hind leg-crippled rats at only 3.3 % of

incidence. We then, loaded the environmental stress on the pups by placing them away from their mothers for 3-4 hr, and then treated them with β-ODAP s.c., mimicking the situation of actual patients to social and physical stresses. The procedure dramatically increased the incidence up to 10 to 35% (average of ca. 25%), although the mortality in the treated rats also increased. Through the autopsy of the dead pups, hemorrhage was always observed at the lower half of their spinal cord parenchyma mainly in the ventral halves, which coincided with the earlier report of Shinozaki H. *et al*. in 1980s (unpublished). The surviving rats showed irreversible paraparesis at their hind legs, dragging themselves without sensory disturbance nor incontinence, and lived over 1.5 years though their body weights were about two-thirds of the controls. Quantitative analysis of 32 segments of their spinal cord revealed that only their lumbar and sacral cord MNs decreased in number reflecting the insult to their hind legs. The remaining MNs showed several pathological changes with chromatolysis, vacuolization, and shrinkage. Considering the report on the ALS model (another MN disease) animals showing hemosiderin deposits in the affected area which was a cause of MN death, our finding of intra-parenchymal hemorrhage could have a role in MN degeneration in our model rats through a similar mechanism[6]. A possible mechanism for the demise of MNs came from our preliminary results *via* increased heme oxygenase-1 activity, which is a contradictory hypothesis on the role of this enzyme to protect tissue from oxidative insults[7].

Another interesting result we have got from our NL model rats was the role of stress on MN diseases. As stated above, stress loading to newborn pups increased the incidence of crippled pups by the β-ODAP treatment. Using this system, we evaluated the level of stress by measuring hormone levels, which are involved in the pathway of stress response i.e. the hypothalamic-pituitary-adrenal axis. The level of both adrenocorticotropic hormone (ACTH) and corticosterone were greatly and significantly increased in the crippled pups treated with β-ODAP. On the other hand, significantly lower increases of these hormones was found in non-β-ODAP-responding groups. The results show that the level of stress response correlated clearly with the incidence of the hind-leg paraparesis in the model. The involvement of glucocorticoid receptors in this pathogenesis was blocked by pretreatment with the glucocorticoid receptor antagonist mifepristone. In addition, dexamethasone, a synthetic glucocorticoid, enhanced the incidence of crippled rats[8]. Our rodent model of experimental NL showed a mechanism of MN degeneration in the spinal cord, and also clarified the disease-promoting effect of the stress. In fact, the outbreaks of NL were often related to the serious social situations such as famine, war and imprisonment. The explanation of the effect of these cases needs more careful analysis. The model, however, revealed that some social stressors could be one of the causal factors that induced experimental NL at extremely higher rates through the enhanced stress-responding system[9].

We are facing global climate change. The possible food shortage because of the decreased crop production may cause the recurrence of forgotten diseases NL and Konzo in under-developed countries. We hope that researchers in this field, including ours, will be mindful of those who have difficulties in obtaining adequate food for their survival. We think that this was the belief and motivation of Prof. F. Lambein's fruitful work in his research.

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Could farmer agronomic practices be contributing to high cyanogenic glucosides in cassava cultivated in konzo-affected areas?

During epidemic and persistent outbreaks of konzo (a cassava cyanide intoxication related paralytic disorder/disease), water stress is often attributed as being a cause of high cyanogenic glucosides in cassava roots. This is of course in addition to the preferential cultivation of high cyanide containing bitter cassava varieties by farmers. High levels of cyanogenic glucosides in edible portions of cassava make cassava unsafe for consumption, as they increase the risk of cyanide intoxication from its consumption. Both drought and dry season water stress have been shown to be able to increase cyanogenic glucosides in cassava roots, but the food insecurity experienced during these periods also makes individuals more susceptible to cyanide intoxication due to the people's poor nutritional status.

Sporadic cases of konzo however also occur, but unlike epidemic or persistent outbreaks of konzo, they can occur independent of water stress. The agronomic causes of increased cyanogenic glucosides in cassava roots during the occurrence of sporadic cases of konzo, is hence unclear. A small study was thus carried out to find out the agronomic factors that possibly contribute to increased cyanogenic glucosides in cassava cultivated in konzo-affected areas. Using cassava root bitterness to reflect cyanide toxicity, the study sought to learn directly from farmers living in konzo-affected areas, the general possible agronomic causes of increased root bitterness in cassava. Being both cultivators and consumers of cassava, the farmers must have made their own observations as to what causes increased cassava root bitterness. The study was carried out in Mtwara region in Tanzania, where konzo was reported to be once persistent. Konzo could still be occurring in Mtwara region even if going unreported.

The farmers pointed out obvious and less obvious possible agronomic causes of increased cassava root bitterness, some of which were independent of water stress and variety type; although they were both mentioned. Soils were mentioned as a contributing factor of cassava root bitterness. The mention of soils was not surprising as soils are generally known to influence cyanogenic glucosides. The specific mention of red soils as a cause of increased cassava root bitterness was however unexpected. Red soils are iron rich and are mainly highly weathered and nutrient poor. Their nutrient poor characteristic related well with the perception that cassava root bitterness additionally arose when cassava was cultivated on soils that had lost their fertility, after being continuously cultivated for a number of years. Increased root bitterness (cyanogenic glucosides) in cassava is hence possibly related to farmers cultivating on nutrient poor soils.

Harvesting cassava early was mentioned as another cause of root bitterness. Plant age at harvest is generally known to influence cyanogenic glucosides in cassava. Young cyanogenic plants are generally known to have higher levels of cyanogenic glucosides than mature plants. Farmers believed that harvesting roots of bitter cassava varieties before 24 to 36 months after planting (MAP) resulted in very toxic cassava roots; this was because they considered bitter varieties as still young plants before 24 to 36 MAP. Cassava however generally matures at 12 to 18 MAP, the bitter varieties were hence specially treated this way because of their perceived toxicity.

Other agronomic causes pointed out as influencing increased root bitterness in cassava included less obvious agronomic practices like poor weeding, branch pruning and piecemeal harvesting. A few recent studies have shown that poor weeding does increase cyanogenic glucosides in cassava roots, hardly any studies have however investigated the effects of branch pruning and piecemeal harvesting on cyanogenic glucosides in cassava roots. The effects of the mentioned perceived agronomic causes of increased cyanogenic glucosides in cassava roots, however needs to be investigated before confirming the significance of the danger they pose to the safe consumption of cassava in areas affected by konzo.

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The QUALATY project

At the end of the year 2015 and the beginning of the international year of grain legumes promoted by FAO (http://www.fao.org/pulses-2016/en/), a multidisciplinary team of Portuguese researchers came to the conclusion that the grass pea (*Lathyrus sativus*) crop could respond to sustainability and health concerns not only of farmers but also of consumers and agro-industries in Portugal. If, on one hand, consumers who are more aware of the influence of food on health, demanded foods that are not only easy to prepare and tasty, but also healthier and more nutritious, on the other hand, agribusinesses sought to produce alternative foods to diversify the food supply, but in a more sustainable way.

However, in Portugal as in the rest of Europe, there was a reduced consumption of grain legumes (and grass pea was no exception), with its cultivation in constant decline, leading to a total national dependence on the outside in what respect vegetable protein (imports of about 70% of the needs in the form of grain legumes) (Vaz Patto & Araújo, 2016).

To reverse this situation, and to make the cultivation and consumption of grass pea a more attractive option, this group of researchers decided to bet on a greater alignment of the objectives of plant breeding and food innovation with the preferences not only of farmers, but also of processors and consumers through more participatory research. However, this alignment of objectives is not always an easy task. For example, several nutritionally beneficial components (such as antioxidants) may give grain legumes a bitter or astringent taste, a common reason cited for consumer disinterest. Additionally these compounds may be associated with greater resistance to diseases or tolerance to water deficit and the alteration of its contents in legumes can cause negative effects from an agronomic point of view (Vaz Patto et al., 2015).

The research project QUALATY (Deciphering the grass pea (*Lathyrus sativus*) quality riddle. How can the omics technologies contribute to a demand-driven improvement in legume quality? ", 2016-2019), coordinated by MC Vaz Patto (ITQB NOVA) and funded by FCT (Foundation for Science and Technology), Portugal (PTDC / AGR-TEC / 0992/2014), was built in an attempt to adapt scientific innovation to the needs and criteria of both farmers and processors as well as consumers, through participatory research.

This project brought together a multidisciplinary team composed of the PlantX lab (https://www.itqb.unl.pt/labs/plantx/welcome) and other ITQB NOVA laboratories (such as the Food Functionality Lab, https://www.itqb.unl.pt/research/technology/Food-Functionality-and-Bioactives), the Municipality (https://www.cm-alvaiazere.pt/) and farmers of Alvaiázere (Simões & Ramos), innovators of the food industry (CookLab) supported by microbiologists (ISA- UL, https://www.isa.ulisboa.pt/) and consumers through sensory analysis (SenseTest, https://www.sensetest.pt/).

With this project, it was possible to better understand the complex genetic control of quality, resistance to biotic and abiotic stresses and production in grass pea and related species (such as Santos et al., 2020; 2018), developing fast and reliable selection methods (associated molecular markers and spectroscopic models, example in Bento-Silva et al., 2019) for the characteristics most relevant to consumers, processors and farmers. These tools will redirect the grass pea improvement so that these characteristics appear combined in new varieties. The project also contributed to the diversification of food products based on grass pea, exploring innovative processing methods (such as fermentation) and applying them into more attractive, convenient and tasty formulations. The sensory analysis carried out paid special attention to children's preferences, since healthy eating habits should start in childhood (example in Rocha et al., 2021). Within the scope of this project, innovation in production systems for environmental diversification and ecological sustainability of grass pea production was also analyzed (example in Sampaio et al., 2020). Pre-breeding plant materials, which include a combination of desirable characteristics of quality, resistance and productivity, are currently being developed. Several works focusing on the diversification of grass pea-based food and on the development of expeditious tools for the future improvement of this legume are also underway and will be published soon.

Undoubtedly, the complementarity of the knowledge developed (fundamental and applied) by the project partners played an essential role in order to be able to achieve the objectives and widely disseminate their results.

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NEWS FROM LAB TO THE FIELD

New video about engineering acyanogenic cassava

CropLife International and the American Seed Trade Association recently released a video about our work at the Innovative Genomics Institute, where we are using CRISPR-Cas9 genome editing to attenuate the cyanogenesis pathway in cassava.

The video describes the motivation behind our project, our approach, and the advantages of genome editing for cassava.

The video may be viewed here, along with further description of our project: https://innovativegenomics.org/news/crispr-cyanide-free-cassava/

(N.B. the footage was taken in a simpler time before masking and social distancing.)

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Prevention of Konzo and other diseases caused by eating bitter cassava

South Kivu which is located in the eastern part of the DR Congo and more particularly the villages of medium plateaus and the Plain of Ruzizi, where the tribes of Bafuliiru, Babembe and Bavira are the majority, cassava constitutes the main food and financial source of the communities. Because of cassava's good adaptation to climatic conditions, its multi-functionality (its leaves are eaten as a condiment, its stem as firewood and its tuber as a staple food), it has been cultivated by more than 80% of the population. It is a source of family financing because after harvest, it is sold to pay school fees for children and to meet other vital needs such as health care, clothing...

Young woman sun-drying the bitter raw cassava for her daily meal

Remember that currently, the farmer prefers to cultivate bitter cassava for many reasons, including high production, disgusting bitterness limits people to access to cassava and esteem by passers-by and other devastators (monkeys, rats, moles, birds, etc.). Bitter cassava constitutes of more than 99% of the production compared to sweet cassava. Again, let us

add that in some places, sweet cassava, following climatic conditions, turns into bitter cassava, which increases the percentage of bitter cassava production, which mainly contains cyanide, a very dangerous product because it is toxic and causes this ignored disease called KONZO.

Despite the importance given to it, this bitter cassava becomes the source of this Konzo disease due to acid it contains. After our 2019 investigations, we found that this Konzo disease kills and the population ignores it. We found that the most affected layer is that of poor peasants living in the villages. They are forced to eat cassava in its raw state with the Sombe (cooked cassava leaves). They cannot afford to consume expensive fish, oil, grains and meats.

The consequences are enormous because consuming the unprocessed cassava can cause vomiting, stomachaches, dizziness, weakness or even death. Paralysis can also continue. Surprisingly, with Konzo, an unknown disease, some people can innocently be accused of witchcraft or bad luck, conflicts generate, and villages are decimated.

Actions to be taken

We find that the main cause of the spread and persistence of this disease is ignorance. It is an unknown disease so it can be avoided if and only if the population takes into consideration the prevention and treatment measures of cassava flour or leaf before consumption.

We recommend that the CCDN Network support us technically and financially to inform health workers and the community about Konzo in

-organizing capacity building sessions with local health workers and community relays on how to diagnose Konzo and prevent it,

- sensitizing local communities on how to prevent Konzo by consuming already processed cassava products. Posters and rural radio broadcasts can help us reach a large number,-providing medical or psychological follow-up (advice) for people or families already suffering or affected from Konzo.

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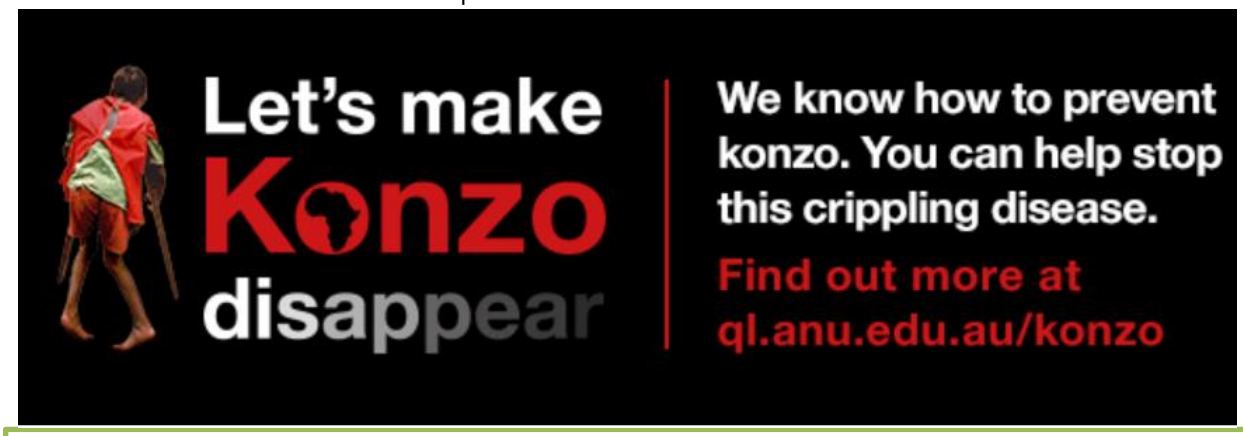

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