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THE POTENTIAL OF SEED GERMINATION INHIBITION TEST FOR EARLY PREGNANCY DETECTION AND IMPROVED REPRODUCTIVE EFFICIENCY OF CATTLE IN ZAMBIA

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ABSTRACT: Early pregnancy diagnosis is an important management practice for reducing calving interval, increasing cattle reproductive efficiency, and the overall herd productivity. This study was undertaken to assess the viability of seed germination inhibition technique (Punyakoti test) for early pregnancy detection in cattle under the tropical rearing conditions. Twenty-four randomly selected cows were used for the experiment. Urine samples were collected and subjected to Punyakoti test, using maize seeds, within 6 hours of collection. Descriptive statistics employing means and standard error were used to analyse data, also, inferential statistics including analysis of variance and t-tests were employed to ascertain differences between the variables under study. Urine from pregnant cows had the highest (80.03±3.99) inhibitory effect while the distilled water group had the least (7.50±3.81) mean seed germination inhibition. The means of germinated maize seeds in pregnant and non-pregnant cow urine treatment groups were significantly different. The means of germinated maize seeds in unstripped and stripped pregnant cow urine were significantly different, while the mean shoot length values for the same treatments were not significantly different. Urine from 42 days' pregnant cows had the highest germination inhibition (80.21±3.59) while the least (25.00±4.35) was observed on day 10. The Punyakoti test reliably detected pregnancy starting from 26 days after insemination. In conclusion, this technique can be used for detecting pregnancy as early 26 days after insemination. The potential role of steroids and involvement of abscisic acid (ABA) in seed germination inhibition requires further investigation.

Keywords: Abscisic acid, Cattle, Maize, Pregnancy detection, Punyakoti test.

INTRODUCTION

Livestock farming including cattle, as a source of meat and income, remains crucial considering that more than 40% of the population in Zambia lack adequate food (MFL, 2020). Due to the need to reduce existing animal protein deficit, programmes aimed to increase production have been developed, these include but not limited to stocking and re-restocking, promoting application of appropriate biotechnologies, and strengthened livestock extension (MFL, 2020). Among the crucial on-farm interventions for improved production is pregnancy detection; furthermore, establishing a pregnancy diagnostic tool allows for the detection of cows that are not pregnant, thus producers are able to make management decisions to increase reproductive efficiency. The decisions may be, culling of infertile females, or re-synchronizing the females that are open (Dilrukshi and Perera, 2009; Balhara et al., 2013; Fontes et al., 2022). When done early, calving interval is shortened thereby increasing the animal's life-time production (Balhara et al., 2013; Aswathnarayanappa et al., 2019).

There are a plethora of techniques which can be used to detect pregnancy in cattle, for example, rectal palpation, non-return to oestrus, radiography, hormonal based assays, and ultra-sound technique (Swamy et al., 2010; Abdullah et al., 2014). Despite their relevance, these techniques, on the one hand, are invasive, costly, laborious, and/or requires a high technical knowhow (Dilrukshi and Perera, 2009) which makes them unsuitable and prohibitive to resource-poor and unskilled rural farmers. On the other hand, Punyakoti test is a farmer friendly, simple, and non-invasive technique also used for pregnancy detection (Dilrukshi and Perera, 2009).

Although a number of studies, focusing on different livestock species with seeds from different cereal species, have recommended its application (Skálová et al., 2017; Aswathnarayanappa et al., 2019; Rahman and Saha, 2020), cattle farmers in Zambia have not yet applied it. Furthermore, there are scarce studies on Punyakoti test with maize seeds. Also, studies including those which used seeds of other cereal species reported variations in the test's reliability, which was attributed to different factors, namely moisture, temperature, daylight, nutrition, and seed storage (Bowden and Ferguson, 2008). Hence, a need exists to validate its applicability in Zambia, utilizing the main and readily available maize seeds, because of its unique tropical climate with unique weather conditions.

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There is also lack of clarity on the urine-containing factors which predispose the seeds to germination inhibition. Over the years, the presence of abscisic acid (ABA) in pregnant cow urine has been associated with reduced seed germination and shoot growth (Islam et al., 2014; Skálová et al., 2017; Rai et al., 2018); coincidentally, some studies have attributed the same to ovarian steroids (Erdal and Dumlupinar, 2010; Lázničková et al., 2020). In view of this controversy, a need exists for further studies to elucidate the potential role of steroids in seed germination. Thus, validation of the actual substance(s) influencing germination of seeds would be crucial in designing simple bioassay kits for use under field conditions. This study was carried out to 1) determine the level of germination and shoot length inhibition for maize seeds in different treatment groups; 2) evaluate the effects of steroids in bovine urine on germination and shoot growths of the maize seeds; and 3) determine the earliest time for detecting pregnancy and non-pregnancy, using maize seeds, in bovines under smallholder farming conditions in Zambia.

MATERIALS AND METHODS

Ethical regulations

Only physically healthy animals were used for this study. The procedures used were non-lethal with minimal to no distress to the animals. The animal handling and experimentation were done with strict supervision by the institutional committee on the animal research, and in compliance with the guide for the care and use of agricultural animals in research and teaching (ASAS, 2020).

Study location and animals

The experiment was conducted at the Field Station, Department of Animal Science, School of Agricultural Sciences, the University of Zambia, Zambia, during the period of June to October 2021. According to the GeoNames geographical database Google Earth-2022, the country is located at latitude S 14° 20' 0'' and longitude E 28° 30' 0''. Additionally, Zambia lies in the tropics, specifically in Southern Africa. The country's average annual precipitation ranges from 800-1400 mm. The temperatures during winter ranges from 10-20 ºC, and during the hot dry season it ranges from 20-30 ºC (RCCC, 2021). The current study was conducted using cattle that belonged to the department of Animal science. Noteworthy, cattle production is the most significant activity, especially among rural farmers in Zambia, compared to other livestock species. Moreover, the highest proportion (93.5%) of the national herd is owned by the smallholder farmers (Odubote, 2022).

Effect of pregnant cow urine on maize seed germination inhibition

A total of 15 pregnant, and 9 non-pregnant and clinically healthy cows were used for the experiment. This study was performed according to the previous study procedure (Swamy et al., 2010) with minor modification including the use of maize seeds. Good quality maize seeds were sourced from a reputable local supplier/research station to ensure better germination efficiency. From these cows, fresh urine samples were collected early in the morning, in clean dry containers, while they were naturally micturating. The samples were subjected to seed germination inhibition test within six hours after collection; each sample was diluted in the ratio of 1:4 from which 15 ml portions were introduced into clean petri dishes, in three replicates, each containing eight maize seeds. As a control, distilled water was also applied to additional petri dishes and nine samples were considered. After 4 days, data was collected for seed germination inhibition % and shoot length on each petri dish.

Assessing the steroids' effect on maize seed germination and growth

Urine samples from clinically healthy, 15 pregnant and 9 non-pregnant, cows were used for this study. The procedure was based on the previous study by Swamy et al. (2010), with modification including stripping the urine samples of steroids. Each urine sample was divided into two subsamples one of which was stripped. Steroid-stripped urine was produced by pouring a urine sample over activated charcoal contained in filter paper held in a funnel. Eight maize seeds were placed on each petri dish, in triplicate, to be grown in the stripped or non-stripped treatment groups. After 4 days, data was collected for seed germination (%) and shoot length (mm) from each petri dish.

Estimating earliest time for detecting pregnancy and non-pregnancy using maize germination inhibition test

The procedure for this experiment was based on the previous study (Rine et al., 2014) with some modification to suite the study objective. Eighteen cyclic Friesian dairy cows were exposed to synchronization using a double prostaglandin ($PGF_{2\alpha}$) injection and were used as either pregnant following insemination (12) or were used as non-inseminated (non-pregnant) hence open Friesian dairy cows (6). For both treatment groups, free-catch urine collection was done at day 10, 14, 21, 26, 30, 35, and 42, every morning and when cows are naturally micturating. Each collected sample was diluted (1:4) and 15 ml of this was introduced into the petri dish (in triplicates) containing eight seeds. The petri dishes were placed on an open area in a store room, and the results were collected after 4 days. Seed germination and shoot length among the treatment groups were determined. Shoot length of each seed was measured (in mm) using a ruler, and the seed germination inhibition (%) was calculated using a formula (Swamy et al., 2010);

$$\text{Germination inhibition \%} = \frac{number\ of\ seeds\ not\ germinated\ in\ petri\ dishes}{total\ number\ of\ seeds\ in\ petri\ dishes}\ x\ 100$$

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Data analysis

The data obtained, for various parameters, was analysed in SPSS IBM® (SPSS IBM 26 version, USA) using descriptive statistics to get the means and standard error of mean (SEM). Furthermore, the differences in the variables under study were determined using one-way ANOVA and the paired t-test statistics. Post hoc (Tukey's HSD) test was performed to obtain the pair(s) with significant mean differences. Significance level (alpha) was set at $P<0.05$. Microsoft excel version 2016 was used to generate the graphs.

RESULTS

Mean seed germination inhibition in distilled water, pregnant and non-pregnant cow urine.

The mean seed germination inhibition for each of the three treatment groups, namely distilled water, pregnant and non-pregnant cow urine are presented below (Figure 1). Urine from the pregnant cows had the highest germination inhibition percent (80.03±3.99) while the lowest (7.50±3.81) was observed for the seeds in distilled water. The analysis of variance revealed significant differences (P<0.05) between the three treatment groups. Additionally, the post-hoc (Tukey's HSD) tests revealed significant seed germination inhibition differences between the pregnant and non-pregnant cow urine groups (P<0.05), pregnant cow urine and distilled water (P<0.05), but not non-pregnant cow urine and distilled water (P >0.05).

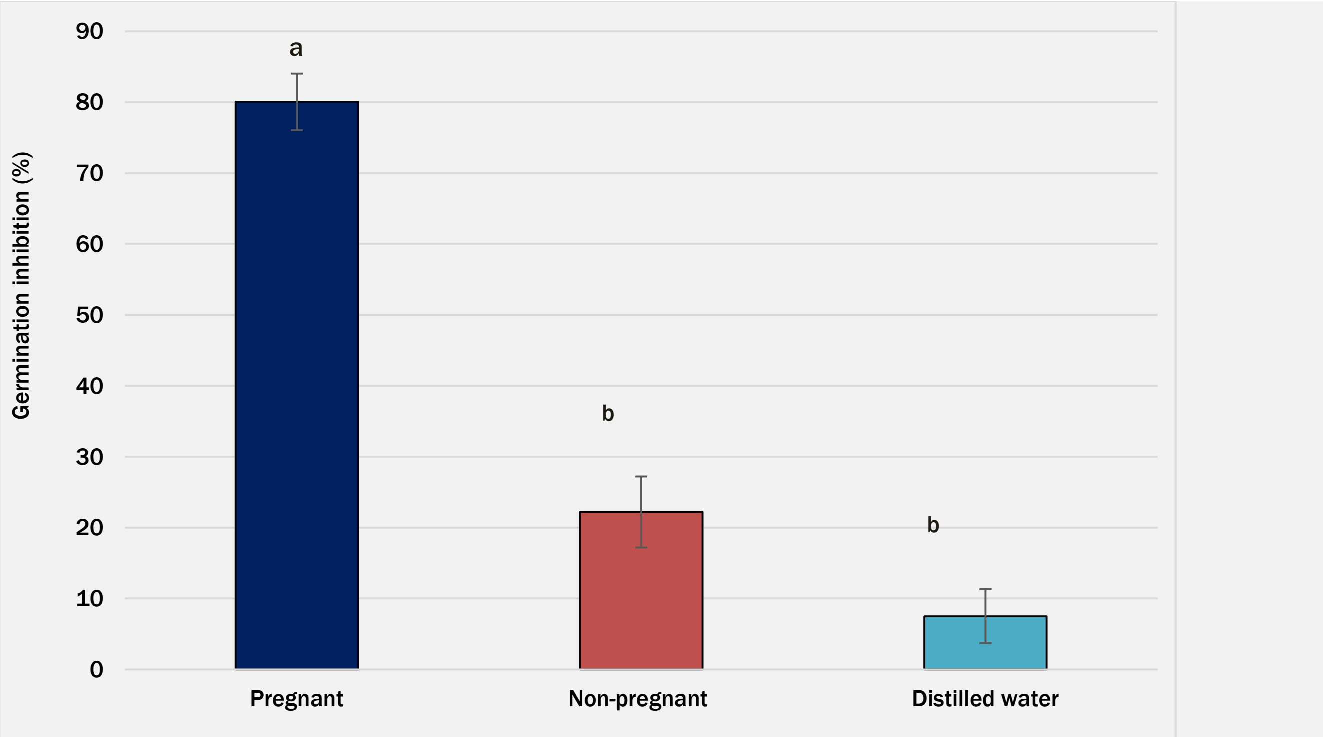

Figure 1 - Mean seed germination inhibition in distilled water, pregnant and non-pregnant cow urine. Different alphabetical letters (A, B) indicate significant difference at P<0.05; Error bars: standard error of mean; %: Percentages.

Mean seed germination inhibition percentage in stripped and non-stripped pregnant cow urine

The mean seed germination inhibition percentage in stripped and non-stripped urine, from pregnant and non-pregnant cows, are presented below (Table 1). The percentage germination inhibition in non-stripped cow urine was generally higher than that in stripped cow urine. The t-test analysis between the stripped and non-striped treatment groups (pregnant) revealed a significant difference (P<0.05) in their mean values; their observed correlation (r) was 0.450. Furthermore, there was no significant difference (P>0.05) in the mean germination inhibition between non-stripped and stripped groups for urine obtained from non-pregnant cows; also, no correlation (r=0.03) was found.

Table 1 - The mean germination inhibition of maize seeds in stripped and non-stripped pregnant and non-pregnant cow urine

Status \ Groups (Treatment)	Non-stripped urine	Stripped urine
Pregnant	80.03±3.99[a]	69.17±5.01[b]
Non-pregnant	22.22±5.01[a]	8.33±3.99[a]

Data expressed as mean±SEM; SEM: standard error of mean; [a,b] means with difference superscripts in the same row are significantly different at $P<0.05$.

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Mean shoot length of germinated seeds in stripped and non-stripped pregnant cow urine

The mean shoot length (mm) of germinated seeds in non-stripped and stripped pregnant cow urine are presented in Figure 2. The mean shoot length of maize seeds in non-stripped urine was higher (5.49±0.84 mm) than that of the stripped urine group (3.35±0.83 mm). A t-test analysis revealed no significant difference (P>0.05) between the treatment groups. Furthermore, no significant (P>0.05) correlation (r=0.020) between the treatment groups was found.

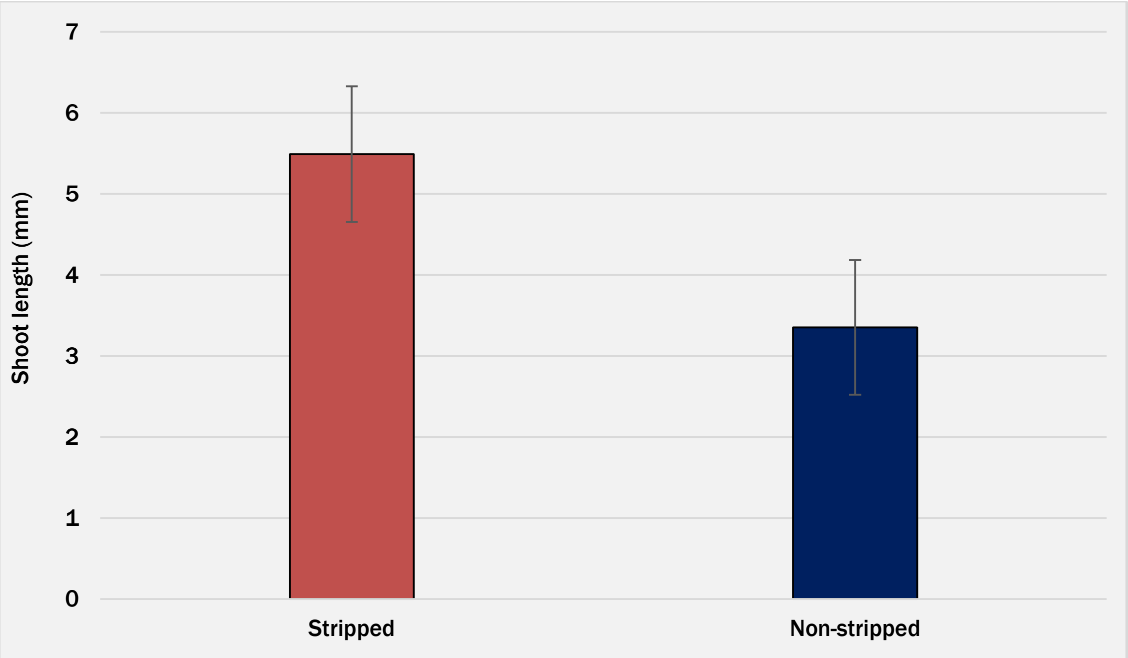

Figure 2 - Mean shoot length of germinated seeds in stripped and non-stripped pregnant cow urine. Error bars: standard error of mean; mm: millimeters.

Mean seed germination inhibition in different groups on different days after insemination

The mean seed germination inhibition in urine from pregnant (inseminated) and non-pregnant cows during days 10, 14, 21, 26, 30, 35, and 42 after insemination are presented below (Table 2). Urine from the 42 days' pregnant cows had the highest mean germination inhibition percentage (80.21±3.59), and that from 10 days' pregnant cows had the lowest inhibition (25.00±4.35). For non-pregnant cows, the highest mean germination inhibition was 29.31±6.18 on day 35, and the lowest mean value (14.58±3.84) was observed on day 10. The analysis of variance indicated a significant difference in mean germination inhibition of seeds in pregnant and non-pregnant cow urine at days 14 to day 42 (P<0.05), but not at day 10 (P>0.05). Generally, the mean germination inhibition, for maize seeds in pregnant cow urine, increased with advancing pregnancy.

Table 2 - Mean seed germination inhibition in different groups on different days after insemination.

Groups (Treatment) / Days after insemination	Pregnant	Non-pregnant
Days 10	25.00±4.35	14.58±3.84
Days 14	33.03±2.81[a]	18.67±5.27[b]
Days 21	46.87±2.25[a]	29.17±5.27[b]
Days 26	55.21±2.86[a]	25.00±7.22[b]
Days 30	77.08±4.57[a]	22.92±5.97[b]
Days 35	66.67±3.56[a]	29.31±6.18[b]
Days 42	80.21±3.59[a]	20.83±5.27[b]

Data expressed as mean±SEM; SEM: standard error of mean; [a,b] means with difference superscripts in the same row are significantly different at P<0.05.

DISCUSSION

Pregnancy detection is an important part of good management which helps to maximise reproductive performance or productivity of cattle and the income of farmers (Dilrukshi and Perera, 2009; Okunlola et al., 2019). It allows for the detection of non-pregnant cows, thus enables farmers to make management decisions to increase reproductive efficiency, for example, culling of infertile females or re-synchronizing the females that are not pregnant (Balhara et al., 2013; Fontes et al., 2022). This shortens the calving interval which contributes to high life-time production and

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productivity in cattle; the costs of keeping non-pregnant cows on the farm without generating income are also avoided through early detection of pregnancy (Swamy et al., 2010; Balhara et al., 2013; Aswathnarayanappa et al., 2019; Okunlola et al., 2019; Fontes et al., 2022). Punyakoti test has been recognized as a door-step on-farm technology by farmers which can be performed at their homes using inexpensive materials with no special skills required (Swamy et al., 2010). Despite its relative advantage (Dilrukshi and Perera, 2009; Swamy et al., 2010), Zambia has not yet popularized this pregnancy detection technique perhaps due to the lack of awareness and/or uncertainty regarding its practicability. This is in view of the previous study which reported merely 8.7% of farmers who were aware of pregnancy detection methods, and that the level of biotechnology awareness influenced farmers' acceptance (Abigaba et al., 2022).

The current findings agreed with those of Okunlola et al. (2019) who reported a high (64.16±2.58) germination inhibition of maize seeds in pregnant cow urine. The significantly higher mean germination inhibition of maize seeds in urine from pregnant cows, for the current study, supports the narrative that Punyakoti test is a viable technique for cattle pregnancy detection in Zambia. It is in support of the previous studies, in other countries, although those studies employed seeds from various cereal species; furthermore, these studies recommended the use of this technique by farmers at local field stations to diagnose pregnancy (Rine et al., 2014; Rai et al., 2018). Of note, our results generally concurred with Okunlola et al. (2019) who reported a significant difference ($P<0.05$) in germination inhibition of maize seeds in urine from pregnant and non-pregnant cows. Various studies have reported similar findings in pigs (Kumar et al., 2017), buffaloes (Aswathnarayanappa et al., 2019), goats (Islam et al., 2014), Ewes (Constantin et al., 2021) and cattle (Skálová et al., 2017; Rai et al., 2018). Coincidentally, and similar to Okunlola et al. (2019), the current study didn't find any significant difference ($P>0.05$) in germination inhibition percentage for maize seeds grown in non-pregnant cow urine and distilled water. Scholars have attributed the higher inhibition effect of urine from pregnant cows to metabolites or other substances in urine, such as abscisic acid (ABA), auxins, and/or ovarian steroids (Hussain et al., 2016; Rai et al., 2018; Lázničková et al., 2020).

Although a plethora of studies have reported significant differences in germination inhibition of various seed species, few studies have focused on, and validated, the same for maize seeds. Moreover, maize is the most commonly grown and readily available cereal crop in Zambia; thus, their use to validate Punyakoti test for pregnancy detection among the most treasured livestock (cattle) in the country (Mumba et al., 2013; Namonje-Kapembwa and Chapoto, 2016) has been long overdue. Similar to the Zambian cattle farming mode, Swamy et al. (2010) reported that most of the times cows conceive by natural mating in their grazing fields and thus the occurrence of pregnancy goes unnoticed by the farmers. In contribution to the early pregnancy detection by cattle farmers, this study has confirmed that Punyakoti test using maize seeds can be relied on, to detect pregnancy in cattle above 26 days after insemination with inhibition of over 55.21%; although, significant inhibition difference ($P<0.05$) between pregnant and non-pregnant cow urine groups was observed from day 14. According to Oknunola et al. (2019), however, urine sample with over 60% germination inhibition effect gives an indication of pregnancy. The current findings also support Rine et al. (2014) who confirmed the test's ability to detect pregnancy by day 28 post-insemination. Considering the economic importance and improved reproductive efficiency of early pregnancy detection (Okunlola et al., 2019; Fontes et al., 2022), utilization of Punyakoti test will greatly benefit cattle farmers in Zambia and beyond.

The increasing germination inhibition with advancing pregnancy period was in agreement with a previous study (Rine et al., 2014). It is plausible that increasing levels of pregnanediol glucuronide (PdG) with advancing pregnancy was partly responsible for the increasing inhibition (Lázničková et al., 2020). Lázničková et al. (2020) previously confirmed that oestradiol-17β (E_2) and PdG were correlated with seed germination and growth. This notwithstanding, other metabolites and substances in urine, such as ABA, and plant auxins have also been associated with inhibition of seed germination and growth (Islam et al., 2014; Rine et al., 2014; Skálová et al., 2017; Rai et al., 2018), although, little is known about changes in their concentrations with advancing pregnancy. What is confirmed, however, is that pregnant cow urine has more levels of ABA than non-pregnant cow urine (Dilrukshi and Perera, 2009; Rai et al., 2018), in addition, a higher concentration of ABA leads to low germination of seed and shoot growth by way of embryo development arrest and inhibition of water uptake (Hussain et al., 2016). Still, there is a need for further studies to establish the changes in ABA levels with advancing pregnancy, and thereby justify the increasing inhibition as pregnancy advances.

As for steroids, some studies reported their influence on seed germination and growth (Erdal and Dumlupinar, 2010; Bowlin, 2014; Lázničková et al., 2020). With the controversy over ovarian steroids and plant growth factors like ABA, this study investigated the potential effect of ovarian steroids, on maize seed germination, by way of elimination approach. The current study found a significantly ($P<0.05$) higher germination inhibition percentage for seeds in stripped and non-stripped pregnant cow urine, which supports the previous reports by Lázničková et al. (2020) and Erdal and Dumlupinar (2010). However, there was no significant difference in the shoot length observed between the two treatment groups. Lázničková et al. (2020) and Bowlin (2014) reported that particular ovarian steroid(s), at different concentration(s), may or may not inhibit germination and/or shoot growth. It is plausible that the inefficiency of the stripping protocol (activated charcoal) used, in adsorbing steroids from urine, during the experiment contributed to the observed effects. On the other hand, the potential contribution, to germination inhibition, from multiple urine-containing factors could mean that only stripping urine of steroids was not enough to cause a significant effect on shoot growth. In light of this, further studies must focus on the proper stripping approach for steroid(s), as well as ABA, auxins and other potential factors to conclude on whether its auxins, ABA, steroids or specific combinations that underlay the Punyakoti test's mode of action.

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CONCLUSION

Punyakoti test is a viable technique for detecting pregnancy in cattle, with over 55% inhibition percentage beyond day 26 post-insemination. This technique relies on seed germination inhibition, caused by urine metabolites such as steroids, to detect pregnancy. Urine from pregnant cows presented higher germination inhibition than that obtained from non-pregnant cow urine or distilled water; the higher observed inhibition percentage levels was perhaps attributed to the ovarian steroids and/or other substances in the pregnant cow urine. To conclusively rule out the effect of steroids on the seed germination and growth rate, further studies are needed to check for the accuracy of the stripping procedure. Additionally, the search for more effective and reliable stripping protocols may prove beneficial. Also, a study on cattle farmers' awareness level of this biotechnology would benefit biotechnology policy formulation and implementation, and guide further research direction.

DECLARATIONS

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Authors' contribution

PC Sianangama conceived and designed the study, supervised, and reviewed manuscript; R Abigaba analysed data and wrote the manuscript; M Mtonga conceived the study, collected data, and reviewed manuscript; SJ Harrison supervised the study and reviewed the manuscript. All authors read and approved the final manuscript for publication.

Conflict of interest

Authors declare no conflict of interest regarding this publication.

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