CIAT Detailed Report (September 1, 2017- August 31, 2018)

1. Introduction

During the previous season CIAT initiated a research for the induction of flowering. A group of six contrasting genotypes were selected and exposed to two kind of treatments: extended photoperiod or red light district (RLD) and plant growth regulators (PGR). Results were promising indicating that plants of most genotypes growing under RLD branched earlier. In two particular clones, the earlier branching also resulted in earlier and more aboundant production of flowers and, eventually, fruits and seeds. The experiments were finalized at seven months of age, thus it was not possible to assess if earlier fruit and seed production could be observed in the other genotypes. PGR experiments yielded positive results as well. Our results suggested that the best responses were observed after the combined application of benzyladenine (BA) and silve thiosulfate (STS). Based on these preliminary results further experiments were planned and executed. The results of the second batch of experiments are described in the present report.

2. Experiments conducted at CIAT during the July 2017-February 2018 period.

Most of the experiments were planted in July 2017 at CIAT Experimental Station in Palmira, Valle del Cauca, Colombia. The materials planted were, again, the same six genotypes used the previous season:

- An “asparagus” clone with sessile leaves (no petiole) and non-branching (**GM 3893-66**).
- The genotype that did not branch, nor flower from the grafting experiment (**SM 3409-43**).
- A genotype that branched but did not flower from the grafting experiment (**SM 3500-2)**.
- The genotype that branched and flowered earlier in the grafting experiment (**SM 3348-29**).
- A late branching/flowering commercial clone (**CM4919-1**)
- An intermediate branching/flowering experimental clone (**GM 971-2**).

2.1 Validation of the effect of photoperiod extension

The same experiment reported in the previous Annual Report was planted for validation during a second season. Each of the six genotypes listed above was planted in single row plots with 10 plants each. In addition two check rows were planted for each genotype. Plants were illuminated with red lights from five individual LEDs all night long. LEDs were placed about 10-20 cm above the growing tip of the plants. As plants grew, the position of the LEDs were periodically moved up to maintain that target distance.

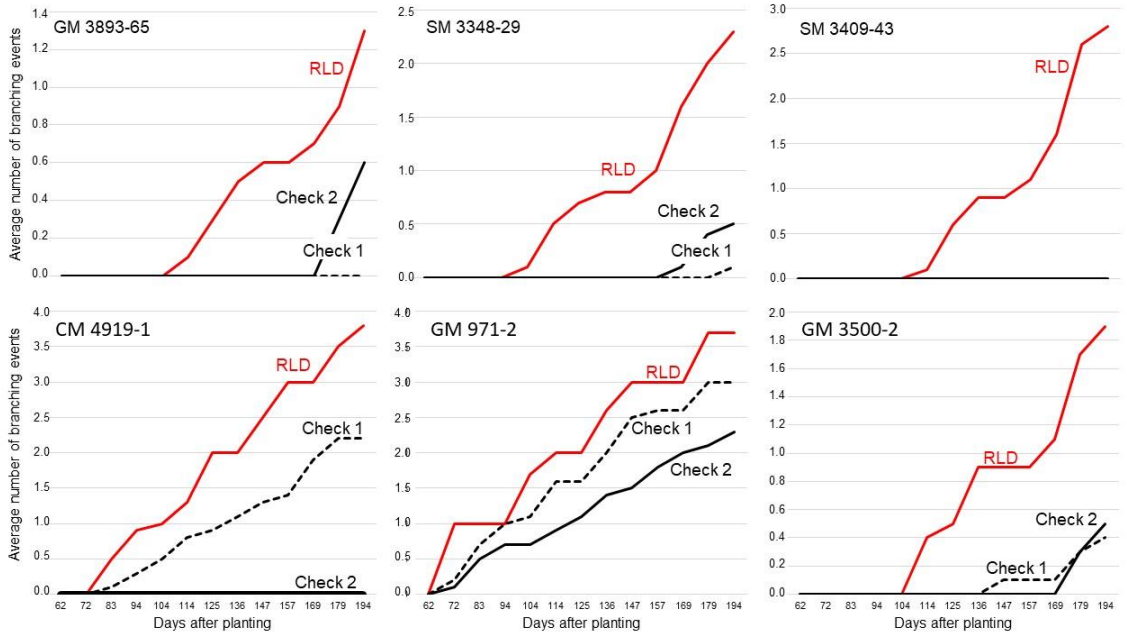
By and large the results from the second season of evaluations confirmed earlier findings. In every case, plants growing under extended photoperiod conditions (RLD) branched earlier and more profusely than the check plots (**Figure 1**). The response variable used in most of this report is the average number of branching events per plant. An average of 2.5 for a given genotype implies that half of the plants had branched two times, whereas the remaining half branched three times.

Interestingly, there was a sharp difference between the checks, particularly of CM 4919-1 and GM 3893-65. In th case of CM 4919-1 the first check plot branched considerably, whereas no branching occurred (as expected) in the second check plot. CM 4919-1 normally flowers for the first time about 8-9 months after planting (MAP). In the case of GM 3893-65 (“asparagous” cassava), which usually flowers when plants are more than a year old, six plants in the second check plot branched about six MAP. No plant in the first check plot of this genotype branched or flowered (as expected). It is suspected that personnel inadvertently collected planting material of the genotypes to be used as checks from plots that had been grown under RLD conditions the previous season. As demonstrated below, a memory (or residual) effect of the flowering induction is transmitted to the following generation.

2.2 Validation of the effect of plant growth regulators

The same experiment reported in the previous Annual Report was planted for validation during a second season using the same set of plant growth regulators (PGR): Silver thiosulfate (**STS**), Benzyladenine - MaxCel (**BA**) and combination of both (**BA+STS**). Because of the results from the

previous season indicaged that a treatment with water and Tween 20 did not elicit any positive or negative reaction in the plants, this treatment was not repeated during this second season. Spraying (as done in the first season) was initiated 75 days after planting (DAP) and at weekly intervals thereafter. As expected, there was some phytotoxic, but manageable effect on some of these treatments. For some reason the phytotoxic effects observed during this second season were stronger that previously observed. In addition, some plots were affected by ants that, in a matter of two days defolitated few plots. The plots for evaluating the effects of PGR were located at the end of the rows and tended to be affected more by occasional water logging as this was the area of land where water drained the last. Plant growth of these plots was not as vigorous as that in other experiments for the induction of flowering described in this report.



Figue 1. Average number of branching events per plant in six different genotypes. For each clone one row was under extended photoperiod conditions with five LEDs maintained about 10-20 cm above the growing shoot. There were two check plots as well. Data from the first checks (particularly for C 4919-1) are supiciuous.

Figure 2 summarizes the results observed during this second season evaluating PGRs. In general results suggest (as was the case in the previous season) that combining the two PGRs provided the best responses (exept for GM 3500-2 and SM 3409-43). In several cases, as exsplained above, the checks branched earlier than expected. This is, for example the case of GM 3893-65 which generally branches when plants are older than a year. In this experiment, however, the second check flowered as soon as six MAP. It should be emphasized that the only genotype that would naturally flower for the first time about 3-4 MAP is GM 971-2. The five remaining genotypes would not flower within six MAP. As illustrated in Figure 2, however, plants treated with PGR began flowering much earlier. In the case of CM 4919-1 between 3 to 4 MAP. GM 3893-65, the "asparagus" cassava began flowering 4-5 MAP. Both checks of GM 3500-2 began flowering around 5-6 MAP (about 40% of the plants), whereas all plants treated with STS had branched by the end of the experiment. SM 3348-29 and SM 3409-43 should not flower as was the case for one of the two check plots. Plants of these two clones treated with STS alone or combined with BA, began flowering six MAP. In general, plant responses to the application of BA alone were disappointing.

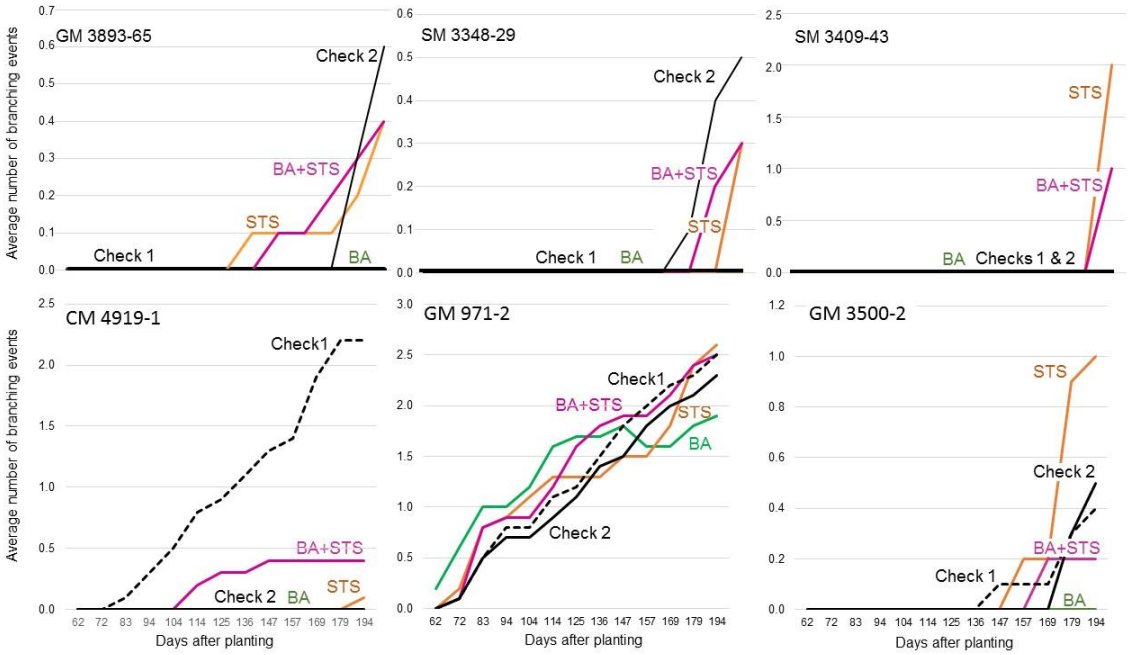


Figure 2. Average number of branching events in ten-plant plots of six different genotypes treated with three different plant growth regulators. In some cases the two checks show very contrasting performances probably because the planting material for one of them was taken from plants that in the previous season had been exposed to stimuli to induce flowering.

2.3 Exploration of the combination of photoperiod extension and plant growth regulators

The same six experimental clones used for RLD and PGR experiments were evaluated in separate plots in which plants were grown under RLD conditions since planting and then treated with the three PGR described above. Each genotype was planted in 10-plant rows. Application of PGR began in 5 of the plants 75 DAP, and 65 DAP in the remaining 5 plants. The main objective of this experiment was to asses if the combination of both stimuli promoted earlier flowering compared with a single stimulus response, or else, if stronger effects could be achieved in "shy" genotypes such as SM 3348-29 and SM 3409-43. Results, however, were disappointing and no clear advantage could be observed combining the two sources of stimulus.

2.4 Exploration of the impact of night breaks on the induction of flowering

Six different night break lengths were evaluated (30, 60, 90, 120, 180 and 240 min) on two genotypes (CM4919-1 and GM 971-2). Breaks took place around 12 PM (e.g. the 30´ break began at 11:45 PM and ended at 00:15 AM). Previous experiences suggested that light intensity (when plants were illuminated all night long) did not have any effect on plant responses. Different sources of light were used for the different lengths of nigh breaks. A maximum light intensity (two 20cm LED tapes) was used for the 90 and 240 min night breaks; an intermediate light intensity (one 20cm LED tape) was used for the 30 min night break; and a low intensity illumination (10 individual LEDs) was used for the 60, 120 and 180 min night breaks. For the all-night long RLD plants were illuminated with the lowest intensity (only five LEDs). There was, therefore, a confounding effect between duration of night break and light intensities.

Figure 3 presents the results from the two clones evaluated. The same information from the check plots presented for the previous experiments was used also here. Therefore, the differences in the two checks, particularly from CM 4919-1, are again observed. There was no clear cut response for the intermediate flowering clone GM 971-2 (left plot in Figure 3). However, an interesting observation could be made. There were two treatments that did not show a better response in comparison with the second check, which probably provides the most reliable information. These treatments are the 60 and 120 min. These are two of the three treaments exposed to low light

intensity. In fact, the third night break treatment with lowest response was the third rwo in which low light intensity had been used (180 min). The best three responses were observed (independently on duration of the night break) in those cases with intermediate to high light intensity.

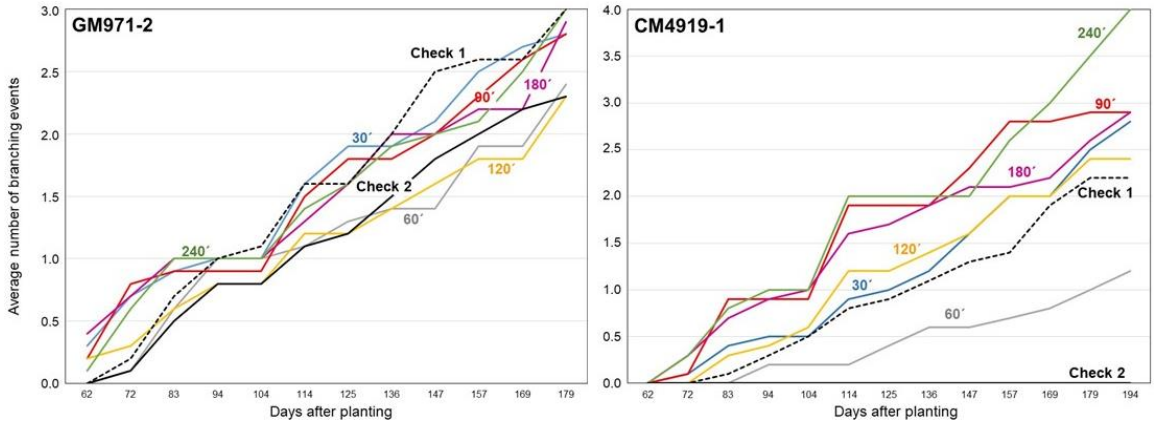


Figure 3. Impact of nigh breaks of different durations (from 30 to 240 minutes) on the two clones selected. For treatments 60, 120 and 180 min, low light intensity was used. For the 90 and 240 min treatments, a high light intensity was used. In the 30 min treatment, plants were illuminated with an intermediate light intensity.

In the case of CM 4919-1 (right plot in Figure 3), responses to the nigh break were much clearer. All treatments (except the 60 min) were better than the two checks. The 60 min treatment was the shortest (time wise) using low intensity light. Results from this experiments show that night breaks are indeed useful inducing earlier flowering. This is important in the case a "portable" illumination system based on solar panels is used. In that case, the amount of energy available may become a bottleneck. This experiments was also interesting because it allowed detecting a clear quantitative response to the stimuli. The longer the night break and/or the higher the light intensity, the better the response from the plants. This quantitative response could not be observed the previous season as different light intensities were used but throughout the night.

2.5 Definition of the minimum light intensity to induce flowering in cassava.

Another experiment was planted to test the usefulness of 5m long LED tapes that were kept at a fixed distance from the ground. In one case, there were four rows and the LED tape was placed between the two central rows at 80 cm from the ground. In a second plot, five rows were planted and the LED tape was placed at 100 cm from the ground immediately above the central row. Each row had 10 plants, five from GM 971-2 and the remaining five with CM 4919-1. In addition to confirming the response of the plants to the light generated by the tapes, the experiment allowed a gradient of light intensity higher in the internal rows, compared with the external rows. A key feature of these plots is that lights were kept in a constant positioning, thus greatly simplifying the implementation of the experiment.

Figures 4A and **4B** present the results from the two plots. In addition to the responses to the LED tapes, these figures also provide the response for the same genotypes presented in Figure 1 ("*Lights on top of the plants*"). Data shown in Figure 1, the lights were low intensity (5 individual LEDs) but were kept at a 10-20 cm distance from the growing shoot of the plant.

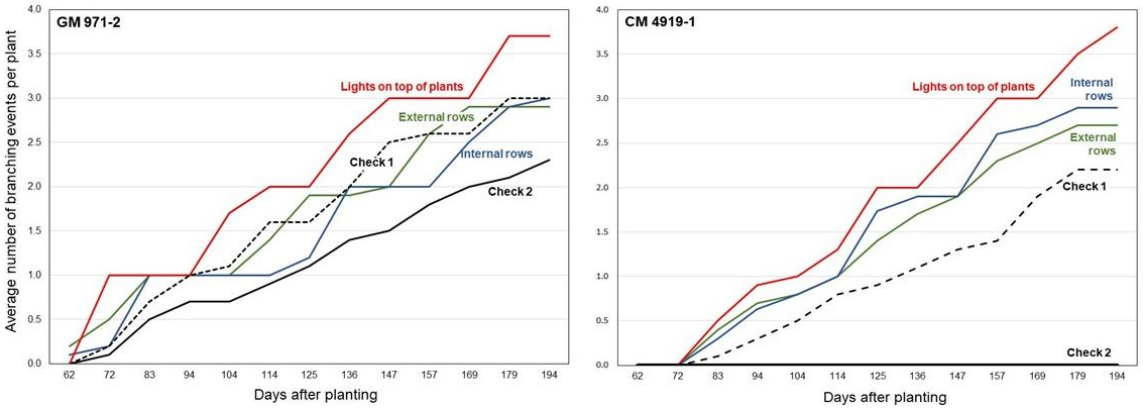


Figure 4A. Response observed in a plot with four 10-plant rows (half with GM 971-2 and half with CM 4919-1) illuminated with a 5-m long LED tape positioned between the two internal rows at fixed 80 cm from the ground.

Results presented in Figure 4A and 4B are consistent with results from the experiments described above. Illuminated plants showed an earlier branching than the most reliable check (# 2). The best responses were observed from plants where the lights were placed 10-20 from the top and moved up as the plants grew (lights on top of plants). In the case of CM 4919-1 the response of plants in the two internal rows was, as expected, better than those in the external rows (**Figure 4A**), but that was not the case for GM 971-2. Differences, howerver, were not large. In the five-rows plot a similar trend was observed (Figure 4B): the best treatment came from lights on top of the plants, followed by plants illuminated with the LED tape and then check # 2. There were, however, no clear differences between plants in different rows within the experiment. It is clear that the illumination of plants in these two plots was above the threshold required to elicit a response which was, nonetheless, lower than that of the lights placed immediately on top of the plant.

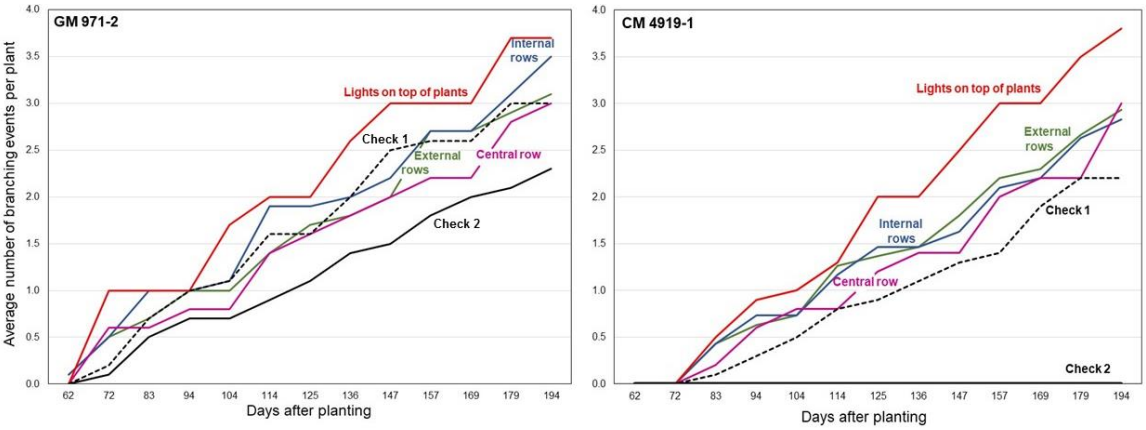


Figure 4B. Response observed in a plot with five10-plant rows (half with GM 971-2 and half with CM 4919-1) illuminated with a 5-m long LED tape positioned on top of the central row at fixed 100 cm from the ground.

The best experiment to assess the light intensity required to elicit a response of plants to illumination with red light during the night was based on the use of a 50W reflector. The experiment included ten rows with a spacing of 1.5m. Each row was 13.5 m long and half of them was planted with 14 plants from GM 971-2 and the other half with 14 plants from CM 4919-1. Spacing of plants within the row was 50 cm. The reflector had a fixed position 3m above the ground on top of the center of the plot (**Figure 5**). In this experiment, there is a clear gradient in light intensity (which is

related to the inverse of the square of the distance to the source). This gradient, however varied as the plants grew. For those plants closer to the center of the plot, light intensity increased as they grew closer to the source. However, for those plants in the periphery of the plot the top of the plant would grow out of the "illuminated cone". Plants at the very periphery of the plot, as expected, branched later than those closer to the center. There was a threshold area (last 2-3 plants in the row, and first and last row in the plot) in which plants flowered at about the same time as the check # 2 (which was planted nearby). There were a group of plants (10^{th} to 12^{th} within each row and the second and the row before the last in the plot (Figure 5) that received about 0.01 µmol m^{-2} s^{-1} at planting time. This very low threshold was enough to elicit already an earlier flowering on both gentoypes (compared with the checks). Plants closer to the center (receiving > 0.03 µmol m^{-2} s^{-1}) flowered even earlier with not much diference among them. The light intensity immediately below the source of light at ground level (e.g. 3 m) was 0.1 µmol m^{-2} s^{-1}.

2.6 Validation of different sources of light for photoperiod extension

The were three different sources of red light (all of them were based on LED technology): individual LEDs (e.g. Christmas lights), LED tapes and the 50W reflector. There was no evidence that plants responded differently to these sources of light. The main response, as explained in the previous sections was the light intensity.

2.7 Validation of the pruning of young branches to induce flowring in cassava

The involvement of CIAT within the first phase of the NextGen project was to work together with GuangXi Subtropical Crops Reasearch Institute (GSCRI) in Nanning. Personnel from this institute had developed an interesting technology to induce flowering in that region of China. Cassava faces a limitation to produce seeds because of the relatively short growing season at that latitude. In August 2017, Hernán Ceballos visited GSCRI and learned the basic technology used to induce earlier flowering. In fact it is a technology that does not induce earlier flowering but rather allows viable fruit and seed set from the first flowering event, which under normal circumstances is sterile. A key feature of the protocol relies on the early detection of the apical meristem shift from vegetative to reproductive growth. The vegetative shoot has a tear shape, whereas the early reproductive shoot acquires a globular shape (**Figure 6**). Soon after this shift the emergence of young branches can be detected and as soon as this is feasible they should be removed (**Figure 6**). Upon the pruning of the young branches the inflorescence assumes the apical dominance which eventually allows fruit and seed set in the first inflorescence.

Only one cultivar (CM 4919-1) was planted in five rows with ten plants each. On top of the first row a 5m long LED tape was positioned. The first row, therefore received a high light intensity which decreased gradually in rows farther away from it. The impact of flowering time (e.g. average number of branches per plant) gradually reduced, particularly for the 5^{th} row. CM 4919-1 typically produces three branches at each flowering event. Some of the young branches were pruned, as soon as they could be distinguished but in the second flowering event (rather than in the first as made in China). Therefore, there were three shoots/plant that could be pruned after the apical meristems shifted to flowering (in the second flowering event). Some shoots were left unpruned while others were pruned for comparison sake. This was done randomly. **Figures 7A** and **7B** illustrate an apical meristem before and after pruning.

There was a positive response in inflorescences from pruned shoots as they grew larger with higher number of flowers and enhanced fruit set (**Table 1, Figure 7D**). A total of 50 plants were planted and 47 were available for this experiment. A total of 191 apical meristems were available from these 47 plants and 122 of them were left untouched while the remaining 69 were pruned. Decision to prune or not was random and, to a some extent, determined by the early identification of young shoots that had already shifted towards reproductive growth. Table 1 summarizes the result of this experiment. The number of fruits and seeds basically doubles in shoots that were pruned versus those that were not.

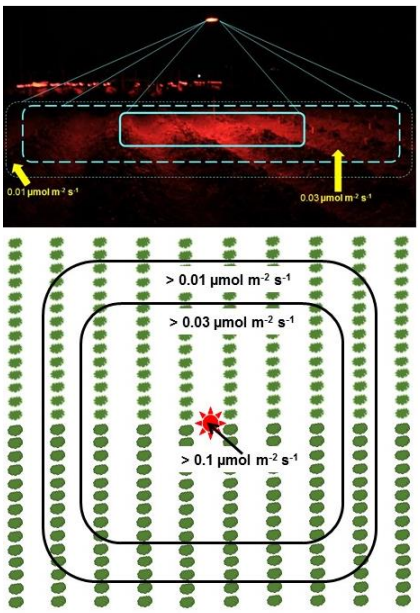


Figure 5. Evaluation of the 50w reflector as source of red light. The reflector had a fixed position 3m above ground. Two genotypes (CM 4919-1 and GM 971-2) were used. In the periphery plants received less than 0.01 µmol m^{-2} s^{-1} of light, which seems to the the threshold to elicit a response from the plants.

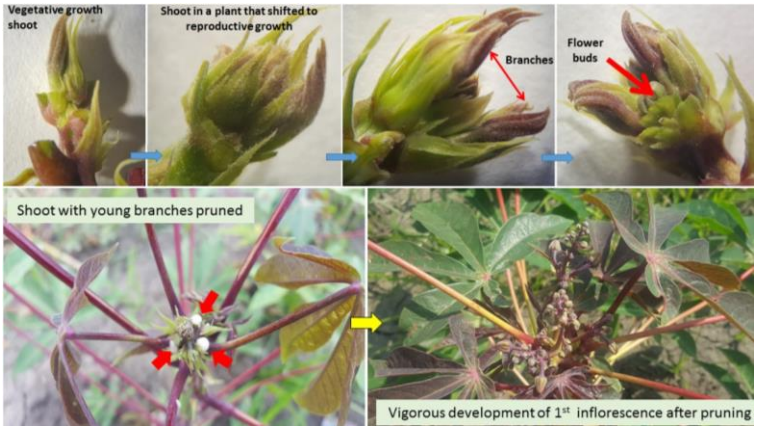


Figure 6. Illustration of the shape change in the apical shoot as it transitions from the vegetative into the reproductive mode (top photographs). Pruning of young branches results in the inflorescence (rather than the branches) excerting the apical dominance.

2.8 Assessment of the memory or residual effect of the induction of flowering

It has been proposed (and earlier interactions with GSCRI supported the idea) that induction of flowering could have a lasting effect if stems from stimulated plants were used as source of planting material. Stems from the two most responsive genotypes the previous season (GM 971-2 and CM 4919-1) were collected and cuttings planted during the present season. Results of this evaluation are presented in **Tables 2** and **3** for genotypes GM 971-2 and CM 4919-1, respectively.

Table 1. Average number of fruits and seeds per apical meristem in pruned or not pruned branches. Data presented for each of the five rows of the experiment. A LED tape with red light was placed

immediately above the first row. Plants in rows further away received less intense illumination and thus showed a fewer number of branching events.

Row	# of plants	Average # of branches per plant	Non pruned		Pruned	
			Fruits (#)	Seeds (#)	Fruits (#)	Seeds (#)
1st	9	4.89	0.22	0.39	0.50	1.13
2nd	10	4.40	0.74	1.70	1.29	3.24
3rd	9	3.78	0.59	1.32	0.75	1.17
4th	9	4.44	1.00	2.40	1.69	3.69
5th	10	3.50	0.85	1.35	1.33	2.47
Across the five rows			**0.61**	**1.27**	**1.22**	**2.61**

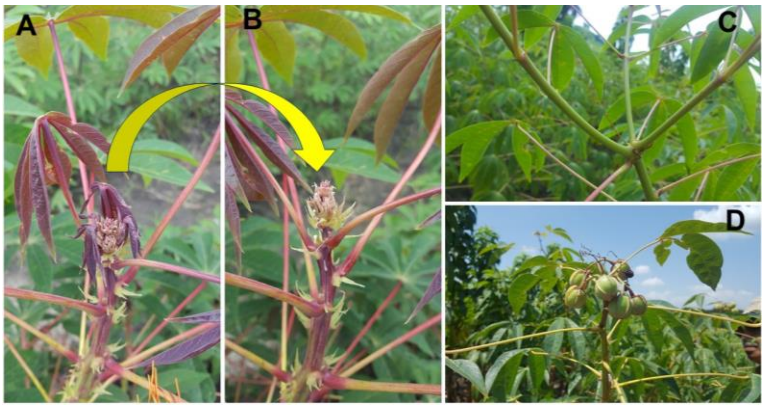


Figure 7. Photographs illustrating the pruning of young branches in cassava soon after flowering can be detected. **A.** An apical meristem in which flowering has been detected soon after it was initiated; **B.** The same shoot after pruning the young branches; **C**. An example of an inflorescence that do not set fruits (frequent in early flowering events); **D**. The effect of pruning on the appearance of to top of the plant showing good fruit set.

Table 2. Results of the experiment to assess the residual effect of flowering induction stimuli through the use of planting material from the stems of treated plants of genotype GM 971-2.

Clone	Treatment of plants from which stem cuttings were taken		Percentage of plants					
			First branching			Second branching		
			Branch	Flowers	Fruits	Branch	Flowers	Fruits
GM 971-2	Check 1		66.7	0.0	0.0	0.0	0.0	0.0
	Check 2		20.0	0.0	0.0	0.0	0.0	0.0
	PGR	BA	50.0	0.0	0.0	40.0	30.0	0.0
	PGR	STS	55.6	0.0	0.0	22.2	0.0	0.0
	PGR	BA+STS	50.0	0.0	0.0	12.5	0.0	0.0
	PGR	Water	0.0	0.0	0.0	0.0	0.0	0.0
	RLD	5 LED	75.0	0.0	0.0	50.0	25.0	0.0
	RLD	10 LED	80.0	0.0	0.0	40.0	40.0	0.0
	RLD	1 Tape	100.0	0.0	0.0	0.0	0.0	0.0
	RLD	2 Tapes	28.6	0.0	0.0	0.0	0.0	0.0

In the case of GM 971-2 no plant branched for a second time on check plants, whereas a considerable number of plants derived from “stimulated” stems branched twice and produced flowers. Production of fruits, however, could not be followed as the experiment was harvested as flowering had just began to occur. Not all stimuli had an impact of the performance of the respective “progeny” but enough differences were observed to demonstrate that, indeed, there is a memory effecto. Results from CM 4919-1 were not as clear as was the case for GM 971-2. Plants from cuttings obtained from BA or BA+STS branched twice and produced flowers in this second branching event. In no case, however, plants produced fruits.

Table 3. Results of the experiment to assess the residual effect of flowering induction stimuli through the use of planting material from the stems of treated plants of genotype CM 4919-1.

Clone	Treatment of plants from which stem cuttings were taken		Percentage of plants					
			First branching			Second branching		
			Branch	Flowers	Fruits	Branch	Flowers	Fruits
CM 4919-1	Check 1		0.0	0.0	0.0	0.0	0.0	0.0
	Check 2		22.2	0.0	0.0	0.0	0.0	0.0
	PGR	BA	30.0	0.0	0.0	10.0	10.0	0.0
	PGR	STS	0.0	0.0	0.0	0.0	0.0	0.0
	PGR	BA+STS	10.0	0.0	0.0	10.0	10.0	0.0
	PGR	Water	0.0	0.0	0.0	0.0	0.0	0.0
	RLD	5 LED	Not available					
	RLD	10 LED	0.0	0.0	0.0	0.0	0.0	0.0
	RLD	1 Tape	0.0	0.0	0.0	0.0	0.0	0.0
	RLD	2 Tapes	10.0	0.0	0.0	0.0	0.0	0.0

3. Scientific presentations and manuscript preparation

The following posters were presented during the GCP21 conference (Cotonou, Benin. June, 2018):

1. L. Marcela Pineda, Nelson Morante, Sandra Salazar, Peter Hyde, Tim Setter, and Hernán Ceballos (2018). Induction of flowering I: photoperiod extension through a red lights district.
2. L. Marcela Pineda, Nelson Morante, Sandra Salazar, Peter Hyde, Tim Setter, and Hernán Ceballos (2018). Induction of flowering II: night breaks as an alternative for photoperiod extension
3. L. Marcela Pineda, Peter Hyde, Tim Setter, Nelson Morante, Sandra Salazar, and Hernán Ceballos (2018). Induction of flowering III: the potential of plant growth regulators
4. L.M Pineda, B. Yu, T. Yinong, N. Morante, S. Salazar, and H. Ceballos (2018). Induction of flowering IV:the potential of pruning young branches

A plenary presentation was also made during the GCP21 conference:
H. Ceballos (2018). Progress and challenges in our understanding of cassava breeding and genetics

At least two manuscripts are under preparation for their publication in peer-reviewed journals.