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Seed-based *in vitro* propagation to accelerate variety development

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Abstract To shorten the current lengthy selection process in sugarcane breeding and to accelerate genetic gain, Sugar Research Australia is implementing a range of novel breeding strategies and selection tactics. One strategy is to rapidly evaluate the progeny of elite crosses in replicated trials without passing through the traditional Stage 1 trials. However, insufficient planting material hinders its adoption. A seed-based *in vitro* propagation system has been developed for sugarcane in which sodium hypochlorite (bleach) and plant preservative mixture (PPM^{TM}) were used in the sterilisation of seeds and seedlings, as well as in the treatment of infected seedlings. The system had been successfully implemented to propagate over 1000 clones of the elite cross Q208^{A} x CP74-2005, for a Stage 2 selection trial. The new system, a first for sugarcane, is more cost efficient, providing three times the number of clones as in the seedling-based micropropagation system with the same input of resources. This innovation will shorten the selection cycle of proven elite crosses by up to 3 years, accelerating the delivery of new varieties.

Key words *In vitro* propagation, seed, breeding

INTRODUCTION

The current Sugar Research Australia (SRA) breeding program utilises three selection stages for variety assessment (Atkin 2012); Progeny Assessment Trials (PAT, Stage 1), Clonal Assessment Trials (CAT, Stage 2) and Final Assessment Trials (FAT, Stage 3). The Stage 1 trials are family trials that test material as single plants in family plots grown from seed. The Stage 2 trials are clonal trials that test a large number of clones planted as stalk segments or setts, from individual plants selected from Stage 1, and are mostly unreplicated. Stage 3 trials are replicated yield trials. Each trial stage provides a source of vegetative planting material for the next trial stage. Using this traditional approach for variety assessment, it takes 10-12 years to release a new variety from the initial planting of progenies of a cross. Stage 1 trials constitute the first 3 years of a normal selection program (X. Wei, pers. comm.): Year 0: crosses are made; Year 1: seeds are germinated, and seedlings are planted as a PAT; Year 2: PAT is harvested, and data collected for selection decision; Year 3: family and within-family selection are conducted, and clones are selected/planted into a CAT or propagated for a CAT.

An elite cross is one that produces a higher proportion of clones entering the later stages of the selection program. In general, a PAT consists of approximately 50% elite and 50% experimental families. Q208^{A} x CP74-2005 is one elite cross in the Burdekin selection program (X. Wei, pers. comm.).

Increasing the rate of genetic gain through breeding and delivering superior varieties that increase industry productivity and profitability is a top priority for SRA. To achieve this goal, novel breeding strategies and selection tactics are being implemented. This includes rapid evaluation of the progeny from elite crosses in replicated trials, bypassing the traditional first 3 years of Stage 1 selection. Insufficient quantity of planting material remains a major bottleneck to implement this novel strategy.

In commercial production, sugarcane is propagated through stem cuttings called setts or billets that normally contain 1-3 buds. Propagation through this method is slow and requires a large amount of planting material. Tissue culture provides an efficient complementary tool for rapid multiplication of sugarcane varieties and its application has accelerated the release of new varieties (Lu *et al.* 2020). Shoot-tip culture and callus culture are the two commonly used methods for sugarcane micropropagation (Lakshmanan *et al.* 2006). However, to generate

sufficient plants from progenies of a cross for a replicated Stage 2 trial, seedlings or seeds need to be used as explants in tissue culture.

To address the issue of insufficient planting material for rapid evaluation of seedling progenies, in the 2018-19 season we first attempted to *in vitro* propagate nursery-derived seedlings. This achieved only partial success as the majority of clones were lost due to microbial contamination despite significant sterilization steps. Based on this, trials on seed for *in vitro* propagation started for the 2019-20 season.

Sugarcane inflorescence consists of florets, either seedbearing or empty. The detached florets retain the glumes and the basal hairs along with other remnants of the shattered inflorescence; this is referred to as “fuzz” by sugarcane breeders (Rae *et al.* 2014).

The first step in micropropagation is the sterilisation of the explant. In this regard, sugarcane fuzz poses a considerable challenge as it easily traps microbial spores in its hairy structure. Different chemicals have been reported for the surface-sterilisation of explants derived from vegetative materials, of which sodium hypochlorite (bleach) and plant preservative mixture (PPM™) are two frequent choices for surface sterilisation (George and Tripepi 2001, Sen *et al.* 2013, Teixeira da Silva *et al.* 2015, Mahmoud and Al-Ani 2016, Zinabu *et al.* 2018, Lu *et al.* 2020). However, there has been no report regarding the use of chemicals for the surface-sterilisation of fuzz.

Here we report the implementation of this type of system for propagating an elite cross, Q208$^{\Phi}$ x CP74-2005, for a CAT yield trial. The trial was designed as a partial replicated trial with 12 or 24 plants required from each clone for one or two replications, respectively. Considering the potential losses in the nursery, we targeted to achieve 30 plants of each of the majority of the clones.

MATERIALS AND METHODS

In preliminary tests during protocol development, we used threshed seeds (glumes and the basal hairs removed) for germination. The results were not encouraging as the germination rate was lower than that of fuzz, although the infection rate was relatively low. Hence, we used fuzz in the later development of the protocol as well as in its implementation. To simplify the text, “seeds” instead of “fuzz” is used throughout this paper.

Sugarcane seeds are very small and vulnerable to any antimicrobial agent. Multiple experiments were conducted during protocol development to test parameters relating to the sterilization and germination of sugarcane seeds with a focus on the choice of chemicals and the duration of treatment. Based on the results, commercial bleach (White King, 4.2% NaOCl) and PPM^{TM} (Austratec) were chosen for the sterilization for both seeds and seedlings.

Seeds from an elite cross Q208$^{\Phi}$ x CP74-2005 were obtained from SRA Meringa, where the cross was made in 2019, and stored at -20°C before use. Germination was conducted at SRA Indooroopilly at weekly intervals for 5 weeks starting from 30 September 2019. A total of 8.3 g seeds was used. On the day of germination, weighed seeds (1-2 g) were placed in a plant growth chamber (Adaptis A1000, Conviron, Canada) at 33°C for 3 hours under light to remove moisture. Then, seeds were sterilised in a 2500 mL flask with a solution containing 1% commercial bleach and 0.2% PPM^{TM} plus 0.05% Tween® 20. The ratio of seeds to the sterilising solution was approximately 1:1000 (w/v). Sterilisation was conducted under room light on a shaker at 130 rpm for 24 hours with two changes of the solution in between. After sterilisation, seeds were washed twice with a solution containing 1% commercial bleach and spread out on a piece of serviette soaked with 15 mL 0.2% PPM^{TM} in petri dishes (90 x 25 mm) under sterile conditions. Approximately 0.1 g seeds (original weight) were placed in a petri dish. The petri dishes were sealed with surgical tape (3M Micropore^{TM}), and the seeds were incubated in the plant growth chamber at 34/32°C (light/dark) under 16 h photoperiod.

After 4 days of germination, the seedlings were removed from the petri dish and washed with a solution containing 0.5% bleach and 0.2% PPM^{TM} plus 0.05% Tween® 20 in a 2500 mL flask for 4 h with one change of solution. The ratio of the washing solution to the seedling was approximately 10 mL per seedling. Seedlings were then rinsed twice in a fresh washing solution before being transferred onto MS basal medium (Murashige and Skoog 1962) either in a 2 mL (seedlings with a leaf not fully developed) or 5 mL (seedlings with at least one leaf fully developed) sterile centrifuge tubes (SSIBio). The medium contained 4.4 g/L MS powder (M519, PhytoTechnology Laboratories) and 30 g/L sucrose with pH adjusted to 5.7 ± 0.1 before being aliquoted into 1L Schott bottle containing 7-8 g agar. After autoclaving at 121°C for 15 min, filter-sterilised PPM^{TM} and 6-benzylaminopurine (BAP, PhytoTechnology Laboratories) were added into the medium at a rate of 0.2% and 2 µM, respectively (referred to as MSPPM^{TM}BAP, or multiplication medium). After 1-2 weeks, seedlings in 2 mL centrifuge tubes were transferred into 5 mL centrifuge tubes containing MSPPM^{TM}BAP, and after 2 weeks in 5 mL centrifuge tubes, seedlings were transferred into petri dishes containing MSPPM^{TM}BAP and sealed with surgical tape. All centrifuge tubes and petri

dishes were placed in a tissue-culture light room at 26°C under 16 h photoperiod of fluorescent lights, about 30 μmol/m^2.s photon flux density at the culture level.

Due to the different response rates of clones to the multiplication medium, different subculture strategies were subsequently used for different clones in order to reach the required number of plants from each of the over 1000 clones within the desired time frame. The majority of the clones had been exposed continuously to MSPPM™BAP with different concentrations of BAP (2 or 4 μM), then continuously to MSPPM™. Some clones became severely phenolic with continuous exposure to MSPPM™BAP and had to be removed onto MSPPM™ for 2-3 weeks and then put back on MSPPM™BAP again.

Starting from late November 2019, 2 months after the start of the germination, clones with 30 or more plantlets in a clump were subcultured into a 630 mL round plastic lunch box container (STATPACK) containing MSPPM™ under sterile conditions. The containers were sealed either with Parafilm®M (Labtek) or kitchen cling wrap. From mid-January 2020, 15 weeks after the start of the germination, clumps of plantlets were separated into single plants and subcultured into petri dishes (90 x 25 mm), initially on MSPPM™, but later on MSPPM™NAA [MSPPM™ supplemented with 2.5 μM α-naphthaleneacetic acid (NAA), Sigma] to encourage rooting. NAA was added into the medium in the same way as for BAP.

Contamination is the single most important factor affecting the *in vitro* propagation process and the quality of the plants. Initially, we tried to achieve complete sterilization without substantially compromising seed viability, but this proved to be unachievable. Thus, we pursued a strategy of contamination management. Basically, the chemical rate and treatment duration in the pre-germination sterilization were kept to a level that allowed most of the viable seeds to germinate and contamination control was integrated into the propagation process. PPM™ was kept present in all the sterilization solutions and all types of culture medium to suppress infection. To avoid cross contamination, seedlings were initially cultured individually in a centrifuge tube. Each infection occurrence was dealt with on a case-by-case basis using various rates and combinations of PPM™ and/or bleach.

Apart from infection, vitrification, somaclonal variation and phenolic browning were other traits affecting the quality of the seedlings. For plants/clones exhibiting hyperhydric symptoms and phenolic browning, they were subcultured and/or the type of medium changed as soon as they were identified. Other than that, no further treatment was carried out due to time and labour constraints.

All plants were checked before dispatching from the lab and clones of poor quality were removed. That included clones severely infected, clones showing somaclonal variation and clones having poor roots including those displaying hyperhydric symptoms.

RESULTS AND DISCUSSION

Germination

During the protocol development, some seeds started to germinate as early as 2 days after being placed in the plant-growth chamber in moistened dishes, while others took over a week and some never germinated. In an attempt to have uniform plants to begin with, each lot of seeds was left to germinate in the plant-growth chamber for only 4-5 days. However, by then some seedlings had already reached the 1.5 leaf stage, some seeds had a tiny green spot indicating the start of germination, while others still had no sign of germination (Figure 1). Contamination was visible as early as 3 days after the start of germination.

Germination rate, expressed as the number of plants per gram of seeds, ranged from 271 to 321/g with an average of 293/g. This was 17% higher than the 250/g germination rate achieved in a subsample of seed from the same inflorescence using the SRA standard procedure in seed-raising mixture (V. Dunne, pers. comm.). Variation between lots in the germination rate was small, demonstrating the reliability of the protocol.

Multiplication

The response to the multiplication medium varied among clones; some were very responsive while others were recalcitrant. This was evident as early as 2 weeks after plants had been subcultured into petri dishes containing MSPPM™ BAP (Figure 2).

Figure 1. Germination of seeds in petri dish after four days of incubation in the plant-growth chamber. Some germinated quickly, while others germinated slowly or failed to germinate. Contamination was visible in nearly all plates. Left panel: all plates in the fifth lot of germination; Middle panel: a close look at some plates; Right panel: a single plate from protocol development.

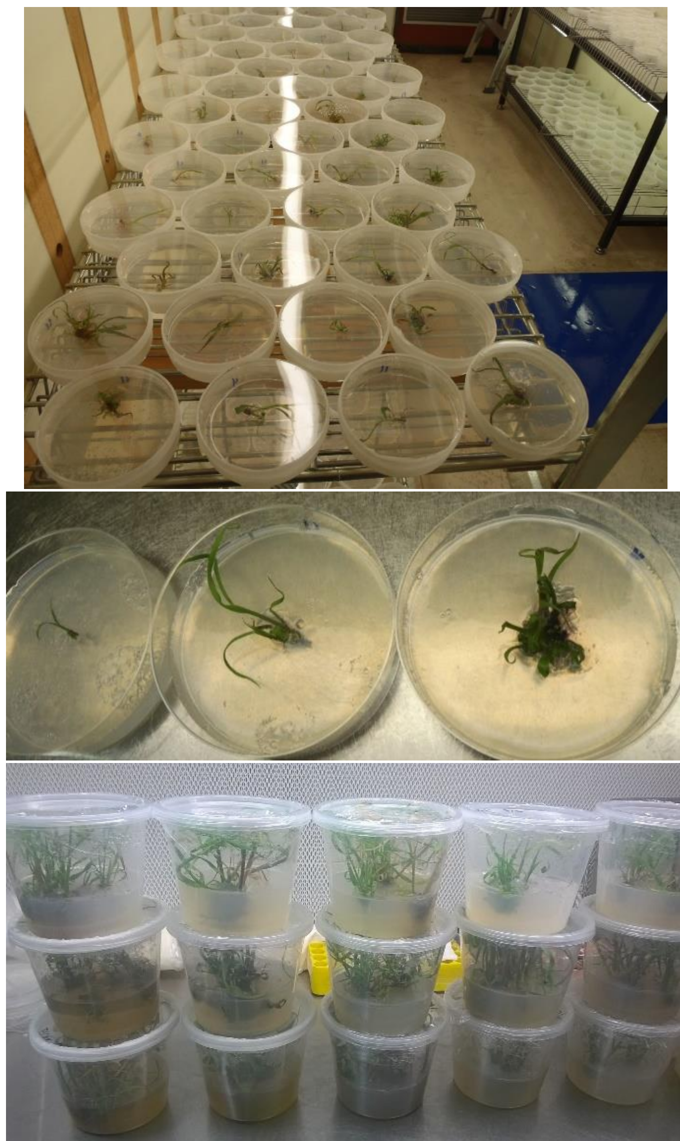

Figure 2. Variation among clones in response to the multiplication medium. Top panel: plates on shelves in the light room 2 weeks after being subcultured, 4-6 weeks after germination; Middle panel: comparison of non-responsive (left plate), intermediate responsive (middle plate) and most responsive (right plate); Bottom panel: plantlets in lunch box containers 20 weeks after germination.

Recalcitrance can be a major limiting factor for *in vitro* plant regeneration, and, despite considerable research, current understanding of *in vitro* recalcitrance and measures to overcome the problems are still limited. Apart from genotype, factors such as culture medium and the frequency of the subculture also affect morphogenesis. In our study, plants were constantly maintained on medium supplemented with PPM^{TM} that might have a phytotoxic effect. George and Tripepi (2001) found PPM^{TM} dramatically reduced the number of shoots formed per explant in chrysanthemum, one of the three species in their study, while the other two species recorded no response. However, we could not exclude PPM^{TM} from the medium due to the frequency of contamination in its absence, given that non-sterile explants were used. To compensate, clones with slow response were subcultured more frequently on MSPPM^{TM}BAP than those responsive ones in order to get the required number of clones ready within a reasonable time frame.

After 8 weeks, about 60% of clones had multiplied into a clump containing 30 or more plants per clone. By then, 10-20% of the clones still had less than five plants per clone. By alternating MSPPM^{TM} and MSPPM^{TM}BAP with BAP at 2 or 4 μM to improve some clones in their responsiveness to multiplication and by increasing the frequency of subculture, we managed to generate sufficient plants for 1130 clones out of 1163 clones that survived the contamination and chemical damages (Figure 4). This allowed nursery planting 24 weeks after the start of germination.

Unlike sorghum (Liu and Godwin 2012) and other cereal species, sugarcane is considered more amenable to plant regeneration with few recalcitrant genotypes (Birch 2014). Our results were consistent with that understanding.

Rooting

Recalcitrance is also true for root development. Clones varied in their response to the rooting medium. It took about 12 weeks for 50% of clones to generate over 30 plants with >5 roots/plant ready for planting in the nursery.

We conducted no experiments on root growth during our protocol development. When rooting started, it was soon noticed that some clones were very slow to develop roots on MSPPM^{TM} medium and plants on MS-only medium (without PPM^{TM}) visually appeared slightly better in rooting. As discussed above, there was evidence of an adverse effect of PPM^{TM} on shoot formation. There was reason to believe PPM^{TM} might also negatively affect root development in sugarcane plantlets, but again it could not be omitted from the medium because of likely reinfection.

We did compare rooting on the commonly used MSPPM^{TM} medium and the MSPPM^{TM} medium supplemented with NAA. The latter was much better for both quantity and quality of the roots (Figure 3). Subsequently, NAA was used in all rooting medium, reducing the time to get clones ready for the nursery planting. Positive effects of NAA on root development were reported both on solid medium (Jamil *et al.* 2017) and liquid medium (da Silva *et al.* 2020) in sugarcane micropropagation. While the NAA concentration in the medium that we used was similar to that reported by Jamil *et al.* (2017), da Silva *et al.* (2020) used NAA at a very high concentration, three times what we used. With this level of NAA in the liquid medium, all tested genotypes produced well-developed root system in a bioreactor (da Silva *et al.* 2020).

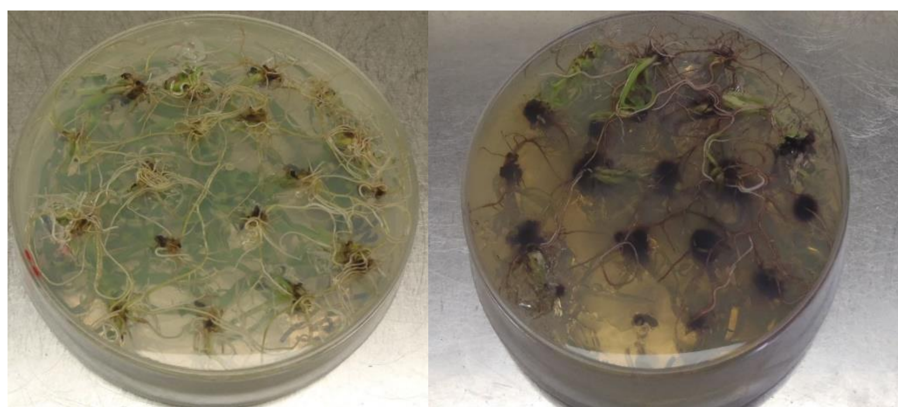

Figure 3. Root development between medium with NAA (Left) and without NAA (Right). NAA in the medium clearly stimulates and improves the growth of roots.

Quality control and success rate

Quality control was seen as a key component in every step of the propagation, aiming to provide healthy and uniform plants for the nursery planting. It started with the planning of the work according to the breeder's requirements, as well as the availability of resources both at the tissue-culture laboratory and the nursery, taking into account likely variation among the clones in their response to multiplication and rooting treatment.

Of the factors affecting the quality of tissue-cultured plants, microbial contamination causes the most serious problems. Contamination not only affects the quality of the plants but also contributes most to the loss of explants or culture. Most infection is caused by fungi or bacteria inhabiting the surface and inside of explants. Therefore, we paid close attention to the sterilization of explants. However, some fungi and bacteria, most likely endophytic, still survived the initial sterilisation. The strategy of integrated contamination management that we adopted proved to be effective. The first key component of this strategy was the constant presence of PPM^{TM} in the sterilisation solution and medium, which suppressed any potential infection. The second key component was to culture seedlings individually in a centrifuge tube after the germination; this effectively avoided cross contamination, thus increasing the success rate.

Starting from 8.3 g of seeds, 2434 clones germinated. One-third of the clones were lost in the first 4 weeks due to contamination (13.8%) and chemical damage (18.6%) (Figure 4). The concentrations of the bleach used in the sterilization of seedlings and contamination treatment might be too high for some seedlings, especially those weak and small ones. As a result, some seedlings became chlorotic or bleached after the sterilization or contamination treatment. The group "chemically damaged" includes not only seedlings lost to chemical damage but also seedlings that had been contaminated and treated with bleach-containing solutions. By week 24 when the propagation had been completed, the cumulative loss due to contamination was 18.0%, while the loss due to chemical damage increased to 34.3%. The loss after week 4 represented 37.8% of the total loss, most likely caused by endophytic bacteria and fungi, indicating the need for further optimization and improvement.

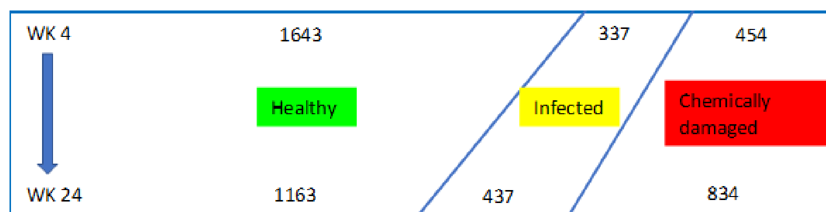

Figure 4. Reduction in the number of healthy plants and the cumulative loss due to infection and chemical damage from Week 4 to Week 24 when the propagation was completed.

Seeds are rarely used as an explant for *in vitro* propagation. We know of only one relevant study, Sen *et al.* (2013), where they used sodium hypochlorite to sterilize the seeds of rough chaff (*A. aspera* L.). They first washed seeds with 75% ethanol for 45 s four to five times and followed with treatments of 1-3% bleach (Clotech, Bangladesh) for up to 25 min. They did not indicate the NaOCl concentration of the Clotech bleach they used. The resulted contamination rate ranged from 36 to 70%, while germination rate ranged from 50 to 60%, both assessed 10 days after sterilization. As a comparison, the initial rate of contamination plus possible chemical damage in our study was at the lower end of their range. The germination rate in our study could not be directly compared with the above results as actual germination rate of sugarcane seeds could not be determined without removing non-seedbearing fuzz.

We devoted considerable effort to saving infected plants, simply because we could not afford losing too many clones. Depending on the type, severity and stage of the infection, either bleach and/or PPM^{TM} were used at various rates and duration. Basically, the treatment was carried out on a case-by-case basis. Despite those efforts, less than 20% of infected plants were saved.

Other factors affect tissue culture quality, including vitrification, somaclonal variation and phenolic browning. While browning constantly occurred, less than 5% of clones showed some degree of a hyperhydric symptom and visible somaclonal variation was negligible.

Browning is a common phenomenon in tissue culture that is caused, largely, by the wounding-induced phenolic exudation. Treatments that have been reported for reducing phenols accumulation in the medium include adding to the medium charcoal, polyvinylpyrrolidone (PVP), NaCl, ascorbic acid and citric acid (Cai *et al.* 2020). Lakshmanan *et al.* (2006) reported that tissue discolouration or browning could be greatly reduced by preparing explants in liquid medium containing ascorbic acid (150 mg/L) and citric acid (100 mg/L).

In our study, browning did not pose as a significant challenge, especially at early stages of the propagation when seedlings were intact. At later stages, phenolic cultures were dealt with simply by changing the medium type and more frequent subcultures.

A previous attempt at initiating the apical meristem of 2-3-month-old nursery-derived seedlings and multiplying through tissue culture (unpublished) gained partial success, as enough plants were generated for only around 350 clones, 25% of the total clones initiated, while the majority was lost due to contamination and chemical damage. Apart from difficulties in sterilization and contamination control, the entire process was tedious and labour intensive. With similar resource input using our seed-based system, enough plants were generated from 1130 clones for the nursery planting and the success rate increased to 46%. Based on an estimated rate of 250 seedlings per gram of seeds (V. Dunne, pers. comm.), there would be 2075 clones germinated in soil from 8.3 g seeds, a success rate would be 54%. However, compared with the 25% survival of progeny achieved by using apical meristem culture from nursery-derived seedlings, we made a remarkable improvement with this novel seed-based *in vitro* propagation system.

Performance in the nursery

Plants were only dispatched from the laboratory to the nursery if they met the criteria of being in good quality, having minimal, if any, evidence of contamination, no visible somaclonal variation, and having good quality roots and not displaying any hyperhydric symptoms.

Plants were sent from Indooroopilly to the nursery in SRA Burdekin between 24 February and 6 April 2020, for deflasking and hardening. The nursery staff reported that they looked as healthy as other tissue-culture-derived plants (C. Kettle, pers. comm.) (Figure 5), indicating that all measures taken during the propagation ensured good quality of the plants.

Figure 5*.* Seedlings in the nursery of SRA Burdekin, 2 months after the planting of the first batch (courtesy of C. Kettle).

Due to space limitations at the SRA Burdekin nursery, only 1046 clones were sent from the laboratory and 1038 clones were planted in the nursery. Of those planted, 90% had enough plants for two replications in a CAT-like field trial. This percentage was higher than the minimum statistical requirement of 25%, as well as the breeder's expectation of up to 50% (X. Wei, pers. comm.). Field planting was conducted in the week of 22 June 2020, 4 months after the planting of the first batch in the nursery (Figure 6). About 40% of clones were planted in replicated plots. At the time of planting, all plants looked strong and uniform within a genotype (X. Wei, pers. comm.).

Figure 6. Field planting in the week of 22 June, 4 months after the planting of the first batch in the nursery (courtesy of C. Kettle).

The trial has been managed as a normal CAT trial. Early assessment of the trial revealed that variation among clones (genotypes) were as expected but, within a clone, plants still looked uniform (X. Wei, pers. comm.). Cane yield (t/ha), commercial cane sugar (CCS) and fibre content will be measured for each plot for the plant and ratoon crops.

There are differences across the clones planted within the propagation process, such as different exposures and resonance time to the different micropropagation steps, and different ages/times from germination. However, no effects from these differences were detected at the time of planting and during the early assessment of field plants. Reducing the duration of the process, especially the nursery planting, would be beneficial in providing a population with minimised introduced differences. We would expect that any effects present at the time of field planting would diminish with each successive crop from plant crop to first-ratoon and even second-ratoon crops.

CONCLUSIONS

Seeds from a sugarcane cross Q208$^{\Phi}$ x CP74-2005 were successfully propagated *in vitro.* Over 1000 clones were multiplied for a Stage 2 selection trial with 90% having enough plants for two replications, 24 plants. This is the first report of such an attempt in sugarcane. This innovation will allow rapid evaluation of progeny of elite crosses in replicated trials without passing through the traditional Stage 1 PAT and will shorten the selection cycle of elite crosses by up to 3 years and accelerate genetic gain. This novel technique will enable SRA to deliver superior varieties faster, hence improving industry productivity and profitability.

The constant presence of PPM^{TM} in the sterilisation solutions as well as in all types of culture medium and keeping individual seedlings separated from very beginning, the two key components in the strategy of integrated contamination management, effectively reduced the occurrence of infection and thus increased the rate of success. However, there was still a substantial loss of clones due to contamination and chemical damage. Further optimization of the protocol needs to focus on the sterilization of seeds before germination. Differences introduced into the process, such as prolonged planting in the nursery, may complicate subsequent selections. The issue needs to be tackled both technically and logistically. Other areas where further studies are needed include the effects of chemicals used in the sterilization and infection treatment on plant growth in the nursery and field, and whether the tissue-culture process and associated use of chemicals bias selection against clones that possess certain traits.

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