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Nicotiana protein kinase 1 (NPK1) in sugarcane: effects on plant development and productivity in Oxisol

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Abstract

Losses in sugarcane crops due to water deficiencies have been increasing in Brazil and worldwide. NPK1 (Nicotiana protein kinase1) is naturally found in tobacco plants and provides tolerance to cold, heat, and salt stress, with positive results in corn, tobacco, and Arabidopsis. This study aimed to evaluate the development and productivity of transgenic sugarcane with the insertion of the tobacco NPK1 gene. Sugarcane plants cv. RB855536 were genetically transformed with the insertion of the NPK1 gene by biolistic (transgenic event obtained by biolistic) and agrobacteria (transgenic event obtained by Agrobacterium infection). These transgenic sugarcane plants were evaluated in relation to the control (non-genetically transformed sugarcane plants). The growth and productivity of the plants were evaluated in a greenhouse, at four periods: 60, 120, 180, and 270 days after planting (DAP). The measurements recorded included the average number, length, and diameter of the stems, number of green leaves, leaf area, and leaf area index. The fresh stalk mass was evaluated at 270 DAP. The insertion of the gene *NPK1* significantly contributed to sugarcane development. There was a significant difference improvement on tillering across all the evaluated periods. At the end of the cycle, plants transformed using the Agrobacterium maintained a higher number of tillers, with 68 % more tillers than the other plants (control and biolistic). The fresh stalk mass was 48 % higher in productivity (than the control and biolistic). Transgenic sugarcane did not show any delay or decrease in growth compared to the conventional non-transformed sugarcane. Therefore, the results suggest that the insertion of the NPK1 gene into sugarcane is a promising alternative for increasing crop productivity.

Keywords: agrobacterium; biobalistic; biometric parameters; biotechnology; growth; hydric deficit, sugarcane drought tolerant, yield.
Abbreviations: NPK1: Nicotiana protein kinase, DAP: days after planting.

Introduction

Sugarcane is grown in more than 120 countries and is a highly important crop producing three main products: ethanol fuel, sugar, and more recently, bioelectricity (Aquino et al., 2018). It stands out that sugarcane is the most important feedstock of sugar production since it provides nearly 70% of the sugar consumed worldwide (Meerod et al., 2021).

Brazil is the largest producer of sugarcane, and the world's largest producer and exporter of sugar, with 41.3 million tons produced and 27.8 million tons exported in the 2020/21 harvest season. This is equivalent to 21 % of the global production and 49 % of global sugar exports. In addition to sugar, Brazil is the second-largest producer of ethanol (behind the United States), with a volume about 36,6 billion liters produced in the 2020/21 harvest season (37% of the total world production) (RFA, 2021; Sica et al., 2020). It also provides biomass to supply 13.8% of the Brazilian electricity demand (MME, 2020).

The total global primary energy supply is mainly derived from fossil fuel sources (oil, natural gas, and coal), with more than 82% coming from this energy source (IEA / OECD, 2014; Silva et al., 2018). 60 % of all oil produced is used by the transportation sector (Silva, 2010). Thus, the increasing global food and energy supplies requires a more sustainable mode of production for diverse sectors. Ethanol is a substitute for fossil fuels to meet these global requirements due to its production efficiency in economic and environmental factors. Thus, the production of alternative and renewable fuels is a global necessity (Aquino et al., 2018).

However, water deficiency is one of the leading causes of productivity losses in sugarcane in Brazil and worldwide, as most sugarcane fields are not irrigated. Losses in the cultivation of sugarcane have been increasing as result of climatic changes occurring in different parts of the world and irregularities in rainfall. Silva and Pincelli (2010) stated that although the stomata can improve water potential of leaves when the crops are rehydrated after the water deficit, they cannot fully recover, because of the complexity in the response which depends on the genotype and drought intensity. This fact influences the breakdown in production. Therefore, the cultivars tolerant to water deficiencies are urgently needed to be developed, not only for maintaining and improving productivity, but also for maintaining the energy security of several countries, including Brazil.

The techniques used in biotechnology can offer alternatives

that reduce the time required to obtain new genotypes and allow the transmission of characteristics (such as tolerance to drought, diseases, and pests) that are otherwise sexually incompatible in plant breeding programs, thus overcoming natural barriers between species.

The NPK1 gene (Nicotiana protein kinase 1) naturally occurs in tobacco plants and provides tolerance to cold, heat, and salt stress. When inserted in Arabidopsis and corn, it protects the photosynthetic machinery of plants against damage caused by drought, thereby improving drought tolerance (Kovtun et al., 2000). The weight of maize grains was similar between transgenic maize without irrigation and non-transgenic and irrigated maize (Shou et al., 2004a) and transgenic maize had an increased tolerance to low temperatures (Shou et al., 2004b) under water deficits. These results demonstrated. the potential of the NPK1 gene in producing genetically modified plants of other species to improve their tolerance of abiotic stresses.

Despite the promising results demonstrated by the NPK1 gene in other crops (corn, tobacco, and Arabidopsis), the effects of this gene on the development and productivity of sugarcane during the harvest season have not yet been investigated. Utilizing the NPK1 gene represents an opportunity to increase production. Our research group previously developed sugarcane plants containing the NPK1 transgene, and maintain stocks in greenhouses for additional research projects (Guerzoni, 2015).

Previous studies have reported that constitutive overexpression of transgenes hinders plant growth, causing decreased productivity (Karim et al., 2007). Under normal or favorable conditions for plant growth, transgene overexpression can compete for a plant's energy and carbon skeletons, reducing the synthesis of proteins and RNAs that are essential for plant growth (Liu et al., 1998).

Thus, it is essential to evaluate the behavior of transgenic plants and their tolerance to stress under normal or favorable conditions for the development of the crop. This study aimed to evaluate the development and productivity of transgenic sugarcane with the insertion of the tobacco NPK1 gene.

Results and discussions

Development of sugarcane plants

The insertion of NPK1 significantly influenced sugarcane in certain stages of development. Stem diameter was not significantly different prior to 120 DAP (Figure 1). At 180 DAP, the plants transformed by Agrobacterium had a significantly smaller diameter (17 mm) compared to the control (21.3 mm) and those transformed by biolistic (20 mm) ($p < 0.05$). However, this result did not persist, by 270 DAP the average diameters were similar between all treatments ($p \geq 0.05$).

There was no significant difference at 60 and 180 DAP in stalk height (Figure 2) ($p \geq 0.05$), suggesting that the transgenic sugarcane caused no delay or decrease in growth when compared to traditional sugarcane. At 120 DAP, the plants transformed using a biolistic were taller (68.3 cm) than those transformed by Agrobacterium (55 cm) and the non-transformed control (58.8 cm) ($p < 0.05$). By 180 DAP, there was no significant difference between treatments ($p \geq 0.05$). This pattern continued until the end of the harvest season (270 DAP), and the plants transformed by Agrobacterium and the control were taller (209.83- and 210.67 cm, respectively) than those transformed by biolistic (198.17 cm).

Landell and Bressiani (2010) evaluated the biometric characteristics of 19 sugarcane genotypes from different regions. They observed that some morpho-physiological processes are strongly affected by some biometric characteristics, while others remained relatively insensitive to the same characteristics. They further concluded that stem length showed the same behavior even under heavy drought conditions, this result is supported by Aquino et al. (2017). Thus, it is possible that the genotypes tested in this study may behave similarly for stalk height, regardless of field conditions.

These results support Shou et al. (2004c), who evaluated 22 genotypes of NPK1 transgenic maize plants in phenological stage R1 under water stress. There was no significant difference between transgenic and control treatments in the height, indicating that the expression of the *NPK1* gene had no negative effect on the growth of maize plants.

Leaf area index (LAI) was significantly different between the treatments at 120 and 180 DAP. Agrobacterium transformed plants possessed the lowest measured LAI (4) than the other treatments (control: 6 and biolistic: 5) (Figure 3). At 180 DAP, the control showed the highest LAI (9.1), followed by agrobacterium (7) and biolistic (7.7) transformed genotypes. The LAI of the transformed genotypes did not differ significantly ($p \geq 0.05$). However, no differences were observed in LAI between treatments at 270 DAP ($p \geq 0.05$).

LAI is important in determining the canopy productivity limits. Any reduction in leaf area and interception of light by the canopy significantly reduces plant photosynthesis. While low LAIs are inefficient in capturing available energy, too high LAIs hindesr growth by shading the lower leaves (Carlesso; Santos, 1998). The ideal LAI is be approximately 4.0, which is sufficient to intercept approximately 95 % of the incident solar radiation. A higher value does not result in a greater absorption of solar radiation (Machado et al., 1982). Oliveira et al. (2007) identified that LAI values above 4.0 are unfavorable for the plant, since they represent an unnecessary energy expenditure, and higher LAI values do not result in higher productivity. Aquino et al. (2017) obtained LAI values between 2.5 and 7 using SP801816 and found that higher LAI values did not result in greater productivity, except in the 4.5 range. Thus, although the Agrobacterium and Biolistic transformed genotypes had lower LAI compared to the control up to 180 DAP, these plants were within the appropriate range for high productivity.

At the end of the harvest season (at 270 DAP) there was a decrease in the LAI values. There was no significant differences between the genotypes, even though Agrobacterium tended to have a lower value (5) than the others (6).

The highest average LAI (9.0) with a subsequent decline at 240 DAP are similar results to those reported by Oliveira et al. (2007), Farias et al. (2008), and Aquino et al. (2017). LAI decreased with a decrease in the number of tillers per linear meter and leaf area per tiller. This phenomenon may be associated with greater competition among tillers (Farias et al., 2008).

Maximum sugarcane tillering was observed at 120 DAP, followed by a decrease in the number of tillers (Figure 4). According to Tavares et al. (2010), the intense tilling phase of the clumps occurs when they reach the maximum production of new tillers, with some varieties producing 20 or more tillers per clump. From the maximum tillering point, the competition between tillers for growth factors (light, space, water, and nutrients) increases significantly, resulting in the reduction of tillering due to the death of younger tillers (Tavares et al., 2010). Thus, after this period of intense growth and competition between tillers, a decrease in LAI is expected because the crop has less metabolic energy available to produce green leaves.

In our experiment, the insertion of the NPK1 gene influenced tillering during all periods (Figure 4). At 60 DAP, NPK1 inserted using Agrobacterium provided greater tillering (6.3 tillers), followed by the treatment using biolistic insertion (3.7 tillers) and the non-transformed control treatment (1.8 tillers). There was no delay or decrease in tiller formation among transgenic sugarcane and traditional sugarcane (control) at the beginning of the harvest season. At 120 DAP, the average of the Agrobacterium transformed genotype remained higher (12 tillers) than the average of the control (5.7 tillers) and biolistic (7.7 tillers) treatments. There was no significant difference between the biolistic and control treatments ($p \geq 0.05$). At 180 DAP, the agrobacterium treatment was still higher (4.5 tillers) than the control (3.7 tillers). At the end of the harvest season, at 270 DAP, the Agrobacterium maintained a higher number of tillers (3.7) with 68 % more tillers than the other treatments (control: 2.2, biolistic: 2.15, tillers)(Figure 4).

Even after the maximum tillering period of 120 DAP, the transgenic plants transformed by Agrobacterium managed to maintain a greater number of tillers (at both 180 and 270 DAP), resulting in greater productivity than the other genotypes at the end of the harvest season.

Among the biometric components associated with productivity, the number of tillers is considered one of the main production components for agricultural potential (Landell et al., 2010). Thus, technologies that increase this parameter provide an important strategy for increasing productivity.

Agricultural productivity of sugarcane plants

The Agrobacterium transformed genotype significantly influenced the fresh mass of the stalks (Figure 5). The agrobacterium treatment showed a higher production of fresh mass (2.5 kg pot^{-1}), compared to the other treatment (control: 1.7 kg pot^{-1} and biolistic: 1.4 kg pot^{-1}). This represents a 48 % higher productivity.

The treatments that provided the highest LAI (greater than 4), during the period of greatest growth by the crop (120 DAP - control and biolistic, and 180 DAP - control), did not result in higher total productivity. This result supports Machado et al. (1982) and Aquino et al. (2017). However, the treatment that showed the greatest tillering (transgenic sugarcane genotype transformed by Agrobacterium) also showed the greatest fresh mass of stalks, which is an essential component of crop production.

The integration of the gene into plant DNA is a complex process that is not fully understood, the site of gene insertion being random and not dependent on homologous recombination. When using bombardment as a transfer method, multiple copies of the transgene can be inserted into the same place in the genome. The insertion of multiple copies of genes can lead to instability in transgene expression, low expression or even gene silencing (the gene is present in the genome, but is unable to express and produce the protein or characteristic) (Kaeppler et al., 2001; Vega et al., 2008).

On the other hand, the use of agrobacterium is of great interest, because it allows the introduction of a low number of copies of transgenes into plant chromosomes, usually one or two (Hiei et al., 1994; Ishida et al., 1996), different from what happens in biolistics, in addition to being considered more efficient and less costly for species compatible with bacteria (Webb and Morris, 1992). Therefore, this may be the reason why transgenic plants transformed via agrobacteria have shown better results compared to the method of transformation via biobalistics.

The *NPK1* gene belongs to the MAPKKK family, which plays a critical role in cytokinesis and is a signaling pathway for auxin and oxidative stress (Kovtun et al., 1998, 2002; Ishikawa et al., 2002). The constitutive promoter (modified CaMV35S) used to insert the gene into the plant is expressed continuously with no need for drought requirement to activate it. This gene is responsible for initiating the metabolic cascade of MAPK-activated protein kinases. This cascade mediates several vital functions in mammals and yeasts, such as cell proliferation, stress responses, and death.

The MAPK cascade consists of three classes of protein kinases organized hierarchically, namely MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). These kinases function as signal transducers integrating information from the cellular environment for the transcription of metabolic responses through phosphorylation. MAPKs are activated by MAPKKs through phosphorylation of the tyrosine and threonine conserved motif Thr-X-Tyr (TXY), which is in the activation loop (T-loop) between catalytic subdomains VII and VIII. Then MAPKKs are activated by MAPKKKs through phosphorylation of serine and the residue of serine/threonine S/TXXXXXS/T (Tena et al*.,* 2001).

MAPK, MAPKK, and MAPKKK homologues in plants have been identified based on conserved yeast sequences and are associated with physiological factors that interfere with productivity. The MAPK signaling cascade is evolutionarily conserved in eukaryotes, including yeast, animals, and plants. Although MAPKKK of alfalfa (MMK3) can be found during all phases of the cell cycle, it functions as a protein kinase only in cells during mitosis, suggesting that it plays a role in the regulation of cytokinesis in plants (László et al., 1999).

A tobacco MAPKKK fragment (*NPK1*) was constitutively expressed in maize through the modified promoter 35SC4PPDK, and its effect on drought tolerance was evaluated (Shou et al*.,* 2004a). The results showed that the photosynthesis rate was maintained under dry conditions in transgenic maize. Moreover, transgenic plants have a similar seed weight to non-transgenic and irrigated plants (Shou et al., 2004a). Similar results were found in rice, in which the insertion of the NPK1 gene increased the tolerance of water stress and induced greater productivity (Ning et al., 2010).

However, some cases have reported a constitutive overexpression of transgenes hinders the growth of plants and may reduce their productivity (Karim et al., 2007). Under normal growth conditions, transgene overexpression may compete for the plant's energy and carbon skeletons, decreasing the synthesis of proteins and RNAs that are necessary for growth. The constitutive expression of genes associated with plant stress response cause slow growth in transgenic plants (Soderman et al., 1996; Liu et al., 1998). The products of these genes are likely to interfere with cell division or activity, resulting in growth inhibition and, therefore, may represent a "stress signal".

Overexpression of *NPK1* in tobacco can have detrimental effects on cell division, embryogenesis, and seed development (Kovtun et al., 1998). Agrobacterium-mediated transformation of maize plants using the vector pSHX004 was reduced due to the death of treated specimens with high expression of the *NPK1* gene during the selection process (Shou et al., 2004a; Muoma; Ombori, 2014).

In our experiment, we did not observe unfavorable results in the development and productivity of plants under normal growing conditions after the insertion of the NPK1 gene in sugarcane. On the contrary, it promoted a significant increase in tillering (68 % higher tillering than the control) and productivity (48 % higher productivity than the control) of the crop. Although all the metabolic processes triggered by this gene (which are associated with productivity) are still unclear, our results are promising for increasing sugar cane production.

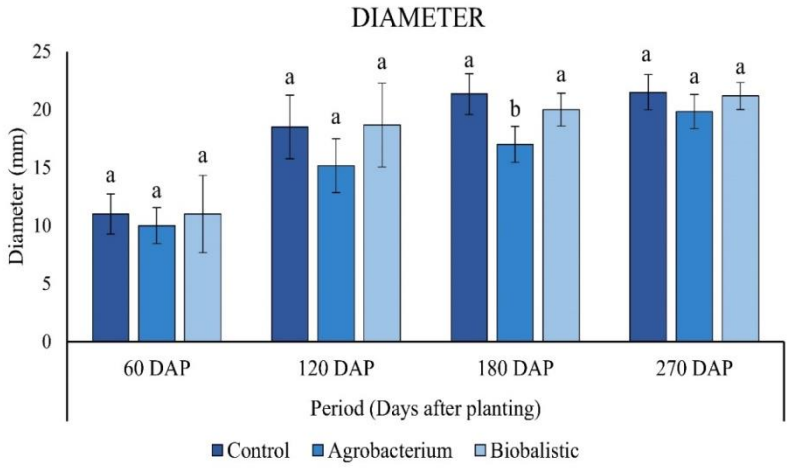

Figure 1. Stem diameter (mm) of conventional sugarcane (control), transgenic sugarcane by Agrobacterium and Biolistic at 60, 120, 180 and 270 days after planting (DAP). Equal letters represent no significant difference between the averages using the Tukey test ($p<0.05$). Source: Authors

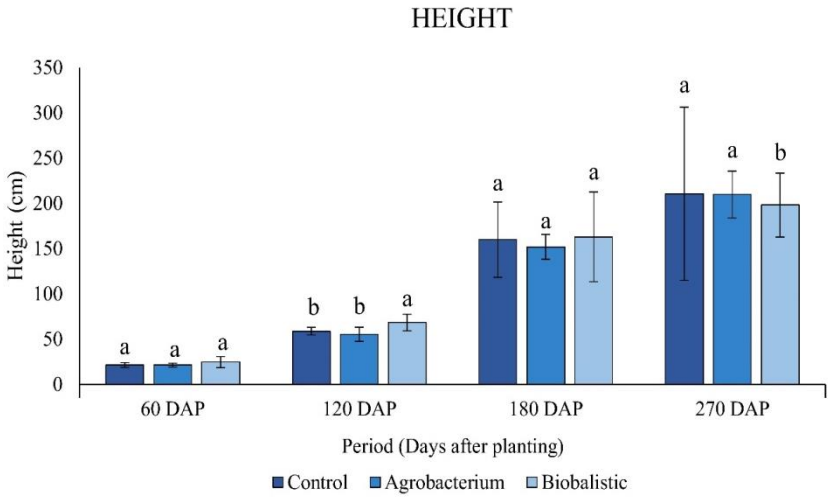

Figure 2. Height (cm) of conventional sugarcane (control) and transformed sugarcane using Agrobacterium and by Biolistic at 60, 120, 180, and 270 days after planting (DAP). Equal letters represent no significant difference between the averages by the Tukey test ($p<0.05$). Source: Authors

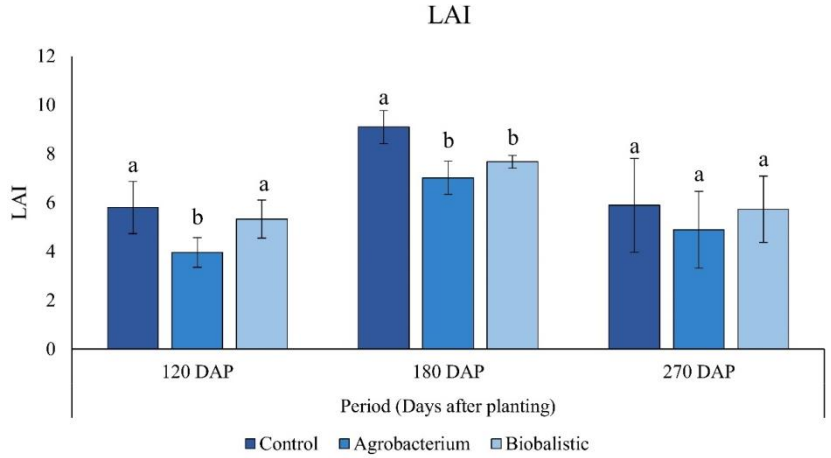

Figure 3. Leaf area index of conventional sugarcane (control) and transformed sugarcane using Agrobacterium and by Biolistic at 120, 180 and 270 days after planting (DAP). Equal letters represent no significant difference between the averages using the Tukey test ($p<0.05$). Source: Authors

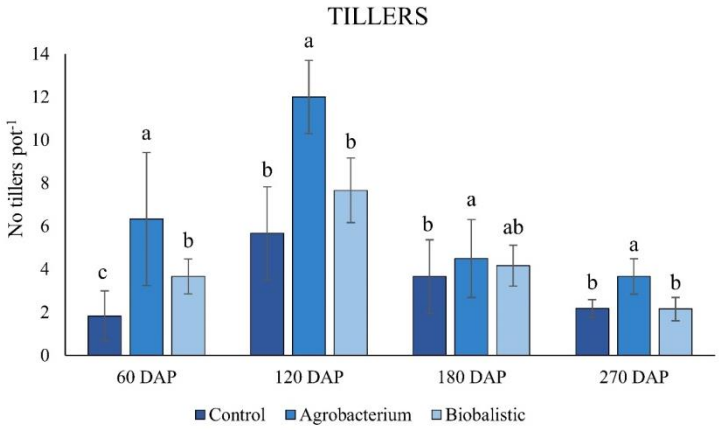

Figure 4. Number of conventional sugarcane tillers (control) and transformed sugarcane using Agrobacterium and by Biolistic at 60, 120, 180 and 260 days after planting (DAP). Equal letters represent no significant difference between the averages using the Tukey test (p<0.05). Source: Author

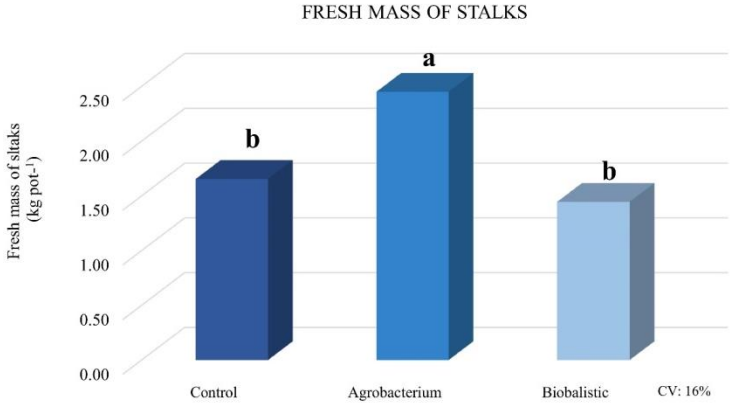

Figure 5. Fresh Mass of Stalks (kg pot^{-1}) of conventional sugarcane (control) and transformed sugarcane using Agrobacterium and by Biolistic. Equal letters represent no difference between the averages by the Tukey method (p<0.05). Source: Author

Some genes have been introduced in sugarcane for the following purposes: tolerance to herbicides (Enriquez-Obregon et al., 1998; Falco et al., 2000; Manickavasagam et al., 2004), resistance to diseases (Enriquez et al., 2000; Gilbert et al., 2005, 2009; Zhu et al., 2011), and pests (Setamou et al., 2002; Christy et al., 2008), and tolerance to abiotic stresses (Zhang et al., 2006; Molinari et al., 2007). Thus, great efforts have been made to improve the productivity of sugarcane in Brazil and the world using biotechnology, which represents energy security in several countries. To date, transgenic sugarcane that is resistant to the sugarcane borer (*Diatraea saccharalis*) has been approved and used in commercial crops. Two new varieties are also expected to be approved (National Agriculture Society, 2018).

It is observed, therefore, that the technological advance in sugarcane is moving in this direction. Thus, the insertion of the NPK1 gene in sugarcane is a promising alternative for increasing crop productivity.

Materials and methods

Plant materials

Sugarcane plants cv. RB855536, aged 6 to 8 months, were used to obtain embryogenic calluses. For this, immature leaves from the meristematic region were used (cylinder of approximately 2 x 10 cm).

Obtaining transgenic seedlings

The entire transformation process was conducted at the Biotechnology Laboratory *(Laboratório de Biotecnologia* - LBI) of the Paraná Agronomic Institute *(Instituto Agronômico do Paraná*-IAPAR) by Guerzoni, (2015), who presents further details of the transformation processes. Sugarcane plants cv. RB855536 containing the Nicotiana protein kinase 1 (NPK1) gene under the control of the modified promoter CaMV35S (35SC4PPDK) (Sheen, 1993) and with the terminator Tnos (nopaline synthase) (Depicker et al., 1982), obtained from Biolistic and Agrobacterium.

For the control were considered non-transformed sugarcane plants cv. RB855536 that passed through in vitro regeneration process following the same protocol used for transformation of sugarcane. After the regeneration of the transgenic plants and control plants, they were acclimatized in a greenhouse for use in this study.

Experimental design

To evaluate the growth and productivity of the transgenic events with the insertion of the NPK1 gene using Agrobacterium and the biolistic method. All experiments were conducted in a greenhouse located at the Laboratory of Biotechnology of the Paraná Rural Development Institute (IDR-IAPAR-Emater), in the city of Londrina, PR, located at latitude 23° 30' S, longitude 51° 32' W, and an altitude of 585 m. The experimental units consisted of 25-liter

polyethylene pots. The soil used in this experiment was classified as *Latossolo Vermelho Eutroférrico* (Eutrophic Red Oxisols). (Embrapa, 2013), eutrophic, clay texture with the following chemical composition: pH ($CaCl_2$) 5.6, Ca (Cmolc dm^{-3}) 8.04, Mg (Cmolc dm^{-3}) 1.46, K (Cmolc dm^{-3}) 1.54, Al (Cmolc dm^{-3}) 0.00, P (mg dm^{-3}) 102, and organic matter (g kg^{-1}) 26.2. The experimental design was completely randomized, with six replicate pots.

The transformed seedlings from biolistic and Agrobacterium (Guerzoni, 2015) were grown in 25-liter polypropylene pots and cut when they reached nine months of age. Stalks containing gems were harvested and cut three centimeters wide gems. After selecting the best gems, they were planted in trays with sand and vermiculite. The trays and substrate were autoclaved prior to use. The plants of the transgenic and non-transgenic events were transplanted to the experimental units (25-liter pots) when the seedlings reached a height of 0.15 m.

The evaluated genotypes of sugarcane cv. RB855536 were AGRO 1 (transgenic plant obtained by *Agrobacterium* infection), CVC7 (transgenic plant obtained by biolistic), and control (non-transgenic).

The development analyses were performed in four periods: 60, 120, 180, and 270 days after planting (DAP). In each period, it was evaluated number of green leaves, leaf area, leaf area index, average stem number, length, and diameter.

The average number of green leaves per stem was determined by counting fully expanded leaves, with a minimum of 20 % green area using leaf +1 (Hermann and Câmara, 1999).

The leaf area (LA) was obtained by measuring the width and length of leaf +1, and then calculated using the equation LA = W × L × 0.75, where W corresponds to width, L is the length, and 0.75 is the correction factor. The leaf area per tiller was obtained using the equation LAT = AF x (N + 2), where AF is the leaf area, N is the number of open green leaves, and 2 is the weighting factor for leaves not yet fully expanded.

The leaf area index (LAI) was determined using the equation LAI = NT × LA/S, where NT corresponds to the number of tillers (m^2), LA is the leaf area per tiller (m^2), and S is the shaded area of the land (m^2) used for the evaluation.

The stems contained in each pot were counted to determine the total number of stems.

The average stem length (m) was obtained by measuring each stem in the pot from the ground level to the first visible auricle (classified as leaf +1) using a measuring tape.

The average stem diameter was obtained using a caliper at the middle third of the stems.

Agricultural productivity

The fresh mass of the stalks was evaluated at the end of the cycle, 270 DAP (March/2017), by collecting the stalks of each experimental unit, removing the green and dry leaves, and pinching and weighing them on a precision scale.

Statistical analysis

The results were statistically analyzed using an analysis of variance (ANOVA), and the averages were compared using the Tukey test. In all analyses a probability value less than 0.05 was considered statistically significant ($p < 0.05$).

Conclusion

The insertion of the *Nicotiana* kinase 1 gene (*NPK1*) in sugarcane significantly influenced the development of the crop. The transformed plants did not show a delay or decrease in growth, and Agrobacterium transformed plants resulted in 68 % higher tillering and 48 % higher productivity compared to traditional non-transformed sugarcane. Our results suggest the insertion of the NPK1 gene in sugarcane is a promising alternative to increasing crop productivity.

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