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Border cell counts of Bollgard3 Cotton and extracellular DNA expression levels

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Abstract

In a world where there is growing pressure to grow more with a smaller environmental footprint, alternative forms of plant protection are needed. The root tips of most plants produce border cells in a mucilage that also contains extracellular DNA (exDNA), which is known to be involved in plant defence. A decade after we first demonstrated that there was varietal difference in the number of border cells in Australian cotton cultivars, we enumerated current commercial cultivars and assessed the level of exDNA produced by individual root tips. The results exposed that there has been a change in the number of border cells per root tip, that cultivar variation still exists and that the recovered levels of exDNA also differs. However, there was no correlation between border cell number and disease resistance to two of the major wilt pathogens and the levels of exDNA did not change when a root tip suspension was incubated with spores of fungal pathogens. The results imply that, while there is potential for border cells and root tip properties to be incorporated into breeding programmes, we still need to develop a better understanding of how root tips are able to influence disease epidemiology if we are to capitalise on this phenotypic property.

Keywords: *Gossypium*, verticillium, fusarium, *Berkelyomyces*,

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Introduction

In the face of a growing global population there exists the requirement for increased food and fibre production, which in the past has largely been delivered from breeding (Morris and Heisey 2003). However, traditional breeding has resulted in a restrictive gene pool, requiring a change in breeding strategies (Holland 2004; Morris et al. 2006; Tripp 1996). In addition to the requirement to increase crop yield, there is also a call to do so with a reduction in the environmental impact that current agriculture practices have (Bauloombe et al. 2009; Epstein 2014). In light of these challenges, one area that is now attracting more research attention is breeding for rooting traits (Paez-Garcia et al. 2015), which offers potential for improved drought tolerance (Carter et al. 2019; Comas et al. 2013; Rabbi et al. 2018), nutrient recovery (Palta and Watt 2009), carbon sequestration (Kell 2011) and disease resistance (Paez-Garcia et al. 2015). Several aspects of root architecture, such as lateral root numbers, rooting depth and root diameter have been linked to potential disease resistance (Snapp et al. 2003; Vieira et al. 2008), but one characteristic of root systems, where a linkage with disease is well established, is border cells (Hawes et al. 2016a).

Most plant species produce border cells, the function of which was initially thought to be protection of the growing root tip by reducing friction with the surrounding soil (Haberlandt 1914). While this function is known to be important (Bengough et al. 2005; Iijima et al. 2000) it is not their primary function. The detached individual and small groups of border cells are instrumental in determining the root associated rhizosphere community, which they achieve through selective carbon (Hawes et al. 1998; Stubbs et al. 2004) and specific signal molecule production and release into the surrounding soil (Baluska et al. 1996; Brigham et al. 1995; Wen et al. 2007). More recently the role of extracellular DNA (exDNA), which has been visualised within the mucilage and potentially coming from border cells (Wen et al. 2017) has been added to the defence roles that these terminally differentiated plant cells have in shaping and managing the rhizosphere (Hawes et al. 2016b; Hawes et al. 2011).

Given the apparent contribution of border cells to root development (Aiken and Smucker 1996), environmental sensing (Brigham et al. 1998) and either disease suppression or avoidance (Hawes et al. 2016a; Hawes et al. 2000; Rodger et al. 2003) it is perhaps surprising that they still appear to receive little to no attention in most breeding programmes. Part of the reason for this may be due to the fact that initial work failed to find much intra-specific variation in border cells (Bozhkov et al. 2007; Hawes et al. 2003), however, variation in border cell numbers has been reported for cotton (Curlango-Rivera et al. 2013; Knox et al. 2007).

The initial work in cotton was conducted with a focus on *Cry* protein expression and the cultivar disease resistance rankings to Fusarium and Verticillium wilts (Knox et al. 2007; Salmond 2003). *Cry* protein expression was detected in the recovered border cell and mucilage suspension, and a moderate positive relationship was observed between resistance to Fusarium wilt and border cell number (r=0.80). In the intervening years, the Australian cotton germplasm has continued to evolve (Downes et al. 2016) and management practices change (Braunack 2013). With the delivery of new Bollgard3™ varieties in the 2016/17 season containing Cry1Ac, *Cry*2Ab and *Vip*3A, further updates to the available F and V rank data and knowledge of the existence of exDNA in the root tip mucilage, we decided it was time to re-evaluate these phenotypic aspects of the cotton root system.

Material and Methods

Cotton seed material

Acid delinted cotton seed of Sicot cultivars 730, 43 Bollgard2 Roundup Ready Flex (BRF), 71 BRF, 74 BRF, 746 Bollgard3 Roundup Ready Flex (B3F), 748 B3F and 754 B3F was provided by Cotton Seed Distributors (Wee Waa, NSW) and used under a material user agreement with Monsanto (now Bayer), Australia. Pea seed of the cultivar Greenfeast and a fodder maize were locally sourced.

Surface sterilisation and germination

Batches of 20 seeds of each cotton cultivar, maize and pea seeds were surface sterilised with a 3 minute wash in a solution of ethanol (50% v/v) and sodium hypochlorite (5% m/v available chlorine), followed by three washes in sterile distilled water. At this time cotton seeds were imbibed for 1 hour, while pea and maize were given 6 hours in sterile distilled water. The surface sterilised seeds were germinated on a layer of moist filter paper laid over 100 ml of water agar (1.5% w/v) in the bottom of a 20 by 10 cm instrument sterilisation tray, which was incubated in the dark at 28°C for 72h.

Border cell enumeration

Border cells were liberated from the root tips by immersing the terminal 15 mm of an emerging radicle in 1ml of sterile deionised water in a 1.5 mL microfuge tube. The root tip was allowed to imbibe for 5 minutes prior to agitation with ten repeated flushes of 100 µL with a pipette. The seedling's radicle was removed and the entire 1 mL suspension transferred to a Sedgewick-Rafter counting chamber (PYSER-SGI, Kent, UK) for enumeration under a compound microscope at 200x magnification. The number of border cells present in 60 arbitrarily chosen cells on the grid were counted and the border cells per tip estimated for all germinated seedlings. This process was repeated for a minimum of three batches of seed for each cultivar.

97 F and V rank data

98 The F and V rank data, which is an indication of the level of resistance a cotton cultivar shows against
99 the wilt fungi *Fusarium oxysporum* and *Verticillium dahlia*, respectively, were obtained from the
100 commercial disease ranking data sets available from Cotton Seed Distributors as of 2018.

101 Extracellular DNA recovery and quantification

102 exDNA was recovered by immersion of the last few mm of a root tip into 30 µL of sterile distilled
103 water within a 1.5 mL microfuge tube. After 10 minutes of imbibition, ten 10 µL aspirations were
104 made to liberate border cells and exDNA from the root tip prior to the root being removed.

105 Evaluation of recovered exDNA

106 In an attempt to remove border cells from the supernatant, solutions other than water were tested.
107 These included solutions of 0.01, 0.1 and 1% pluranic acid as a surfactant, SDS buffer (0.1% sodium
108 dodecyl sulphate 0.1% (w/v) in 100 mM ammonium acetate), ¼ Ringer's solution and TBE buffer at
109 5x (54 g Tris base, 27.5 g boric acid dissolved in 900 mL of deionized water to which 20 mL of 0.5 M
110 EDTA (pH 8.0) was added before making up to 1 L) and 1x strength solutions. These recoveries of
111 exDNA were also conducted either with or without centrifugation at 7000 rpm (2400 x g) for 10 and
112 30 seconds in a bench top centrifuge, in an attempt to pellet border cells and leave exDNA in
113 solution. To ensure viability of the border cells post recoveries, 20 µL of the mucilage and border cell
114 suspension was transferred to a dimple slide to which 2 µL of SYTOX™ green was added, mixed with
115 a pipette tip, and the border cells visualised under UV and bright field light microscopes to
116 enumerate both total and dead cells.

117 Quantification of exDNA was initially undertaken using both a Nanodrop 8000 microvolume UV-Vis
118 spectrophotometer (Thermo Scientific) and the broad range Quant-iT dsDNA Assay Kit (Invitrogen)
119 with a SpectrMax M2 (Molecular Devices). Quantification on the Nanodrop involved measurements
120 taken from 2 µL of the recovered solutions having blanked the machine against the specific recovery
121 solution. Quantification with the Quant-iT kit was conducted as per the manufacturer's instructions,
122 which briefly involved adding 10 µL of our unknown sample to 200 µL of their BR buffer in a 96 well
123 plate, loading the dsDNA BR standards and determining fluorescence with the SpectrMax M2 after
124 mixing for 30 seconds and with excitation/emission set to 485/530 nm.

125 Fungal spore preparation

126 Cultures of *Fusarium oxysporum* f. sp. *vasinfectum* and *Verticillium dahlia* isolated from infected
127 cotton plants were obtained from Dr. Linda Smith of the Queensland Department of Agriculture and

Fisheries. A culture of *Berkeleyomyces rouxiae* (formerly *Thelaviopsis basicola* isolate 40192) were obtained from the late Prof. Lily Pereg of the University of New England, Armidale. Sub cultures of these were prepared on ½ strength Potato Dextrose Agar (Oxoid, UK) with chloramphenicol present at 10 µg/mL. Fungal cultures were developed for 7 days at 25°C at which time a 0.5 x 0.5 cm section of the growing colonies mycelial front was aseptically removed and transferred to 100 mL of Potato Dextrose Broth (Sigma-Aldrich, USA) in a 400 mL baffled Erlenmeyer flask. These liquid cultures were grown at 25°C, 150 rpm for two weeks. At this time fungal spores within the suspension were estimated by removing 100 µL of culture, which was made up to 1 mL with deionised water, loaded into a Sedgwick Rafter slide for counting under 200x magnification. This process was repeated five times for each culture and the potential colony forming unit per µL of solution was estimated.

Determination of fungal presence on border cell extracellular DNA secretion in cotton

Border cells and exDNA were recovered as described for the cotton varieties Sicot 730, 71 BRF, 714B3F, and 748 B3F, and maize and pea. To the resultant 30 µL border cell suspensions, 5 µL of one of the pathogen cultures was added, vortexed and incubated at 25°C. exDNA was determined from 2 µL of these suspensions on the Nanodrop 8000 at time 0, 24 and after 48 hours after inoculation. This experiment was repeated three times with root recovery from four seedlings each time.

Results

Cotton border cell counts

Border cell counts for the cultivars Sicot 730, 43 BRF, 71 BRF, 74 BRF, 714 B3F, 746 B3F, 748 B3F and 754 B3F were estimated at 9839, 6612, 9593, 13126, 16716, 10027, 8142 and 6079 border cells per root tip, respectively (Figure 1). Pea border cell counts averaged 3180 per root tip with 2772 border cells/root tip for the maize cultivar.

Relationship between border cell numbers and F and V rank

F rank and V rank data, in addition to the varieties previously assessed was only available to add to the original analysis for Sicot 730, 43 BRF, 71 BRF, 74 BRF, 746 B3F, 748 B3F, 714 B3F and 754 B3F (Figure 2). Little to no correlation existed for these varieties between border cell number with either F or V rank (r = 0.43 for both). Adding this data to the original data set removed any evidence of the relationship that had been previously observed between Fusarium wilt and border cell number (Knox et al. 2007).

Extracellular root tip DNA

Analysis of the recovered root tip exDNA indicated a difference between cotton varieties (Figure 3). Both the Quant-iT and Nanodrop methods of exDNA determination produced good standard curves against the Invitrogen standards, between 5 and 40 ng/µL, with r>0.99. However, DNA quantification with the Quant-iT kit returned very low DNA values when compared to assessments of water recoveries and determination with the Nanodrop (average of 2.59 versus 2110 ng/µL, respectively). Addition of pluranic acid, ¼ Ringer's and TBE at both 1 and 5x concentrations lowered the DNA recovery compared to the water control, although not significantly (*P*=0.31). SDS buffer gave slightly higher DNA recovery than water, although also not significantly (*P*=0.31), but border cells recovered into SDS were found to be less viable (95 versus 100%) when stained with SYTOX green.

Attempts to separate border cells from the exDNA with centrifugation proved to be ineffective in most cases. The amount of exDNA recovered in the supernatant was generally significantly less (*P*<0.001) regardless of surfactant or time of centrifugation. The only exception was with SDS buffer, where 96% of the water control (without centrifugation) exDNA levels could be recovered after a 10 second centrifugation.

Effect of fungal presence on border cell extracellular DNA secretion

Microconidia, conidia and some hyphal fragments of *F. oxysporum* were recovered from the liquid PDB culture at ~5000 cfu/mL. *V. dahlia* was recovered at ~750 cfu/mL and consisted mostly of conidiophores, although some microsclerotia were present. *B. rouxiae* presented 1860 cfu/mL, mostly as single and double celled chlamydospores with only a few epiconidia observed. The 5 µL additions of the fungi to the border cell and mucilage suspension therefore represented approximately 25, 5 and 10 cfu per reaction for *F. oxysporum*, *V. dahlia* and *B. rouxiae*, respectively.

There was no measurable change in border cell suspension exDNA levels in response to the presence of fungal spores and conidia at 0, 24 or 48 hours post exposure.

Discussion

Cotton border cell counts

The original assessment of border cells of Australian cotton cultivars noted that the counts for Sicot 189 were in keeping with those previously reported for cotton (Hawes et al. 2003; Knox et al. 2007), however, there was a considerable gap in the border cell counts between Sicot 189 and the other cotton varieties, which had considerably fewer border cells. The enumeration of border cells of varieties commercially released in the intervening decade produced a range from approximately 6000 to 16000 border cells per root tip (Figure 1), filling the original data gap and extending the range beyond what was previously recorded. This observation highlighted that there remained

variability in the border cell production of different cultivars, while allowing the potential disease relationship, previously observed, to be investigated further.

Relationship between border cell numbers and F and V rank

Despite the newer varieties providing border cell counts in the range where there were previously gaps, the relationship between border cell numbers and F and V rank was weakened with the incorporation of the new data (Figure 2). The apparent lack of relationship between border cell numbers and Verticillium and Fusarium wilts is not surprising, given the epidemiology of these fungi is known to interact with the plant as it develops (Rodriguez-Galvez and Mendgen 1995), implying that these fungi have the potential to penetrate a more developed root. This is supported with experimentation using green fluorescent protein tagged Verticillium that has demonstrated the fungus colonising cotton above the root tip (Zhang et al. 2013), which could imply some form of suppression by border cells, mucilage and exDNA at the root tip.

Extracellular root tip DNA and effect of fungi on recovered levels

With the role of border cells now widely accepted as going beyond that of easing the physical progression of the root tip through the soil environment, attention has turned to their role in environmental sensing and pathogen avoidance. More recently, the properties of the mucilage that surrounds the border cells has been investigated (Bozhkov et al. 2007; Hawes et al. 2016a). One of these properties is the presence of extacellular DNA, which appears like a net within the mucilage and has analogous functions to aspects of the mammalian immune system (Hawes et al. 2011; Wen et al. 2009).

Our experiments highlighted limitations in the detection of exDNA using a diagnostic kit, which we assumed to be due to the presence of peptide and carbohydrate in the mucilage (McCully 1999). We were unsuccessful in our attempts to use either surfactants or buffers to improve recovery of exDNA or to segregate the exDNA and border cells from the mucilage suspension with centrifugation. We did note that current Australian cotton cultivars had a much lower level of root tip exDNA than maize, which resulted in ~14000 µg DNA/tip, with a standard error of ~3000 µg DNA (n=10), significantly higher than the exDNA we recovered from the cotton root tips (Figure 3) despite having fewer border cells per root tip.

Incubation of root tip recoveries with fungal inoculum had no discernible impact on the level of detected exDNA. The lack of response to Fusarium and Verticillium propagules may again be due to the epidemiology of these fungi (Rodriguez-Galvez and Mendgen 1995). However, the lack of response to *B. rouxiae*, given its interaction with cotton as the seedling develops (Nehl et al. 2004),

was unexpected. Possible reasons may well be that the border cells, mucilage and exDNA recovered from recently germinated seedling rapidly changes in the soil environment as the root develops, which is plausible given that differential expression is already known to occur in border cells as they develop (Brigham et al. 1995; Hawes et al. 2016a). Additionally, it may have either been an issue with the relatively low fungal inoculum levels used, that there is no initiation of a defence response (Gunawardena et al. 2005), or that perhaps the border cells are not the source of the exDNA. While it has been demonstrated that root tip exDNA is produced from live root tip cells (Wen et al. 2017; Wen et al. 2009), the site of production has not been confirmed. Experiments involving incubation of root tip recoveries, including those reported here, have not produced changes in detectable levels of exDNA (Wen et al. 2009). Additionally it is acknowledged that DNA synthesis is one of the first processes to halt as border cells develop from the root cap initials and progress into the columella (Phillips and Torrey 1971). As such, it is conceivable that root tip exDNA may be a product of either apical or root cap initial cells. Explanation as to why exDNA is observed in close association with border cells (Wen et al. 2017) may be due to either the retrotransposon like nature of the exDNA (Wen et al. 2009) or the involvement of proteins expressed in the root tip secretome (Wen et al. 2007). Whatever the reason, this highlights one of the current challenges with exDNA research, in that it remains difficult to separate border cells from the mucilage and the associated exDNA.

While the quantification and variation of exDNA (Figure 3) might signify opportunities for further development in plant defence strategies, potentially offering development via changes in expression levels or even in the coding of this genetic material (Wen et al. 2009), the development of this will likely occur in the private sector. The technology user agreement under which we have to access to Australian germplasm we are currently prohibited from investigating the plant derived exDNA further. The changes between publicly and privately funded breeding programmes is already known to have an impact on progress and development (Morris et al. 2006). It may well come down to whether there are perceived financial returns to be gained from further exploration and development of cotton's exDNA as to whether this aspect of potential plant defence, in a time where pesticides are becoming more limited, is explored further.

Conclusion

It is often said that the only two certainties in life are death and taxes, but in the face of an increasing global population and a rise in calls for less environmentally damaging agriculture, then new plant protection tools need to become another of life's certainties. There is little doubt that border cells and root tip exDNA have a role in plant protection and there is potential for border cell screening in plant breeding programmes. However, our understanding of the mechanisms and the

differential expression of signal molecules and exDNA needs to advance in order to fully capitalise on these plant phenotypic properties.

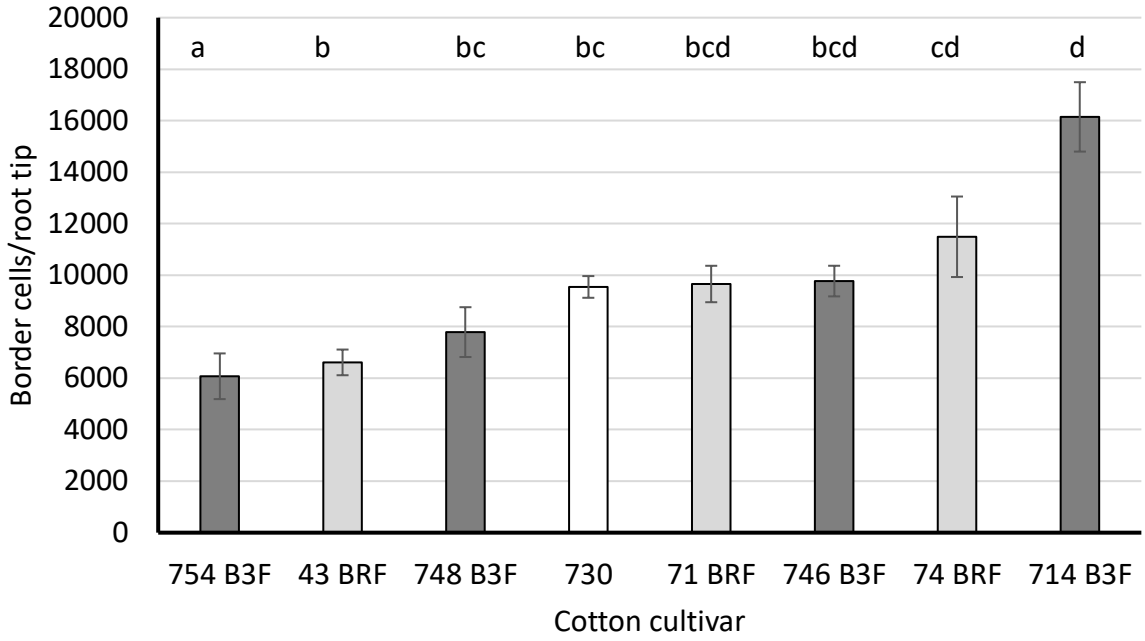
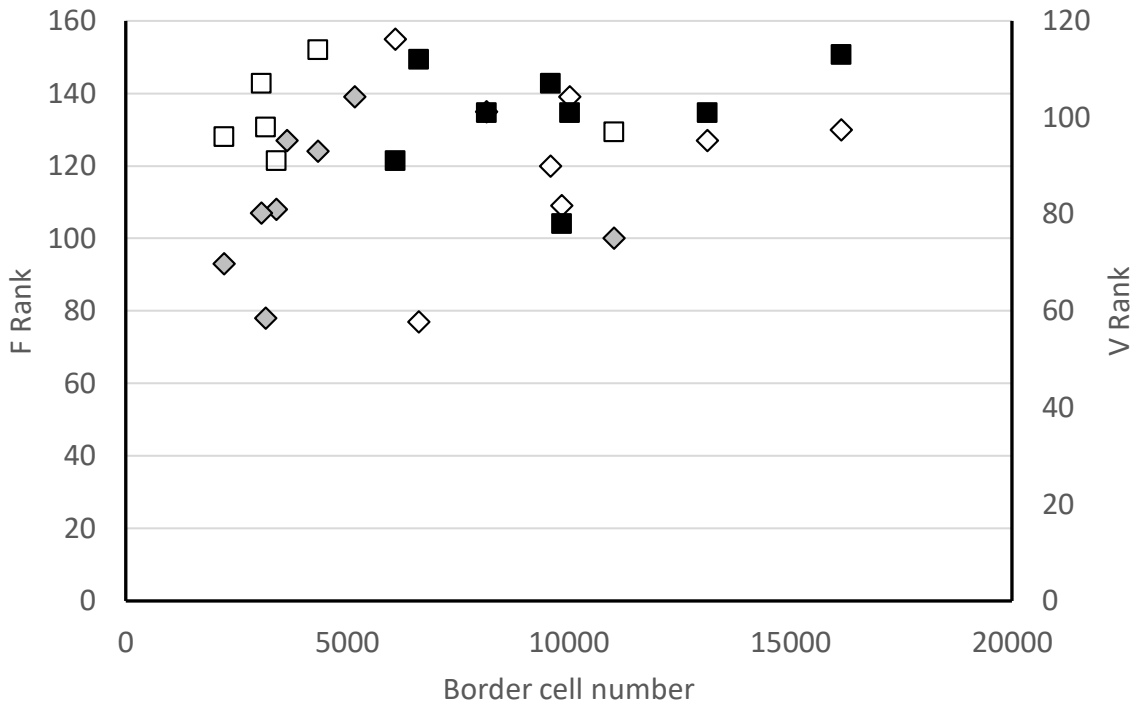


Figure 1 Mean border cell counts per root tip of Australian Sicot cotton varieties. BRF is short for BollgardII™ Roundup Ready Flex (light grey) and B3F for Bollgard3™ Roundup Ready Flex (dark grey). Sicot 730 is a non-GM conventional cultivar. Error bars indicate the standard error of the mean (n between 12 and 21). Significant (P<0.05) differences are indicated with different lower case letters, based on an unbalanced ANOVA and Tukey's 95% confidence intervals .



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267 *Figure 2* CSD trial Fusarium Rank (F Rank, diamonds) and Verticillium Rank (V Rank, squares)
268 data plotted against mean border cell number per root tip for the varieties assessed in 2005
269 (filled) and 2016 (open). An F or V rank of 100 is assigned to the background level of
270 resistance, so above 100 is more resistant to disease.

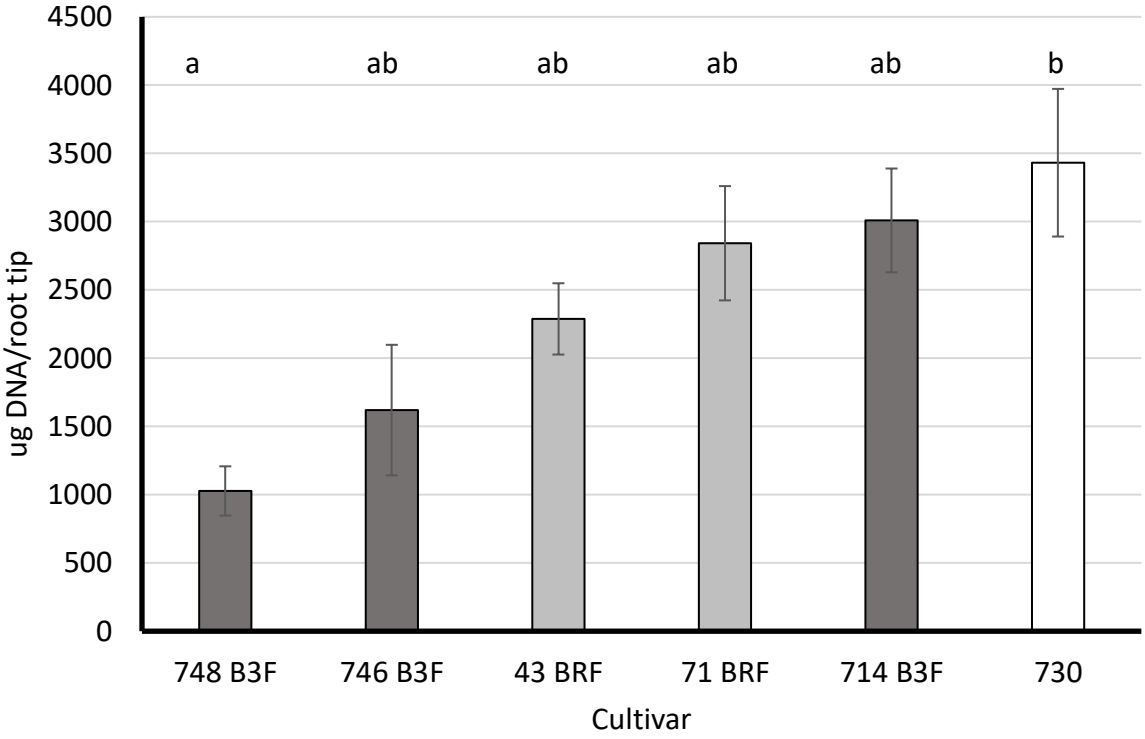

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Figure 3 The mean water based recovery of exDNA in root tip mucilage and border cells of Sicot cotton varieties. BRF is short for BollgardII™ Roundup Ready Flex (light grey), B3F is Bollgard3™ Roundup Ready Flex (dark grey) and Sicot 730 is a conventional cultivar. Error bars are the standard error of the mean (n between 9 and 23). Similar means, based on an unbalanced ANOVA and Tukey's comparison, are indicated with similar lower case letters.

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