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Biochemical basis of resistance to pod borer (*Helicoverpa armigera*) in Australian wild relatives of pigeonpea

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Abstract

The domestication of pigeonpea has severely impacted the intrinsic host-plant resistance (HPR) to pest and diseases, particularly pod borer (*Helicoverpa armigera* hubner). This study with 41 Australian wild *Cajanus* genotypes and interspecific hybrids demonstrated a high level of resistance to *H. armigera* in the accessions of *Cajanus acutifolius*, *C. latisepalus*, *C. lanceolatus*, *C. pubescens*, and *C. reticulatus var. reticulatus*. Significant variation in herbivory development and mortality ($P < 0.001$) was observed in the wild accessions and their hybrids in response to feeding on leaves. A strong positive relationship ($R^2 = 0.69$, $P < 0.001$) between total phenolic compounds (TPC) and the HPR was observed. Australian wild genotypes demonstrated the role of TPC and the absence of certain flavonoids such as rutin and quercetin in resistant genotypes. The detached leaf bioassay technique separated the wild and domesticated accessions into wild resistant, with herbivory weight difference (HWD) (Day 7–Day 1) ranging between −27 - 104 mg, wild susceptible, with HWD ranging between 124 - 207 mg and domesticated susceptible, with HWD ranging from 208 - 300 mg. Similarly, based on TPC, accessions were also categorised into wild high TPC, with TPC ranging between 32.3 - 42.5 GAE mg/g DW, and wild low TPC had only 17.2–24.8 GAE mg/g DW. Low TPC concentrations were found in domesticated pigeonpea, with 10.7–17.6 GAE mg/g DW. The presence of very high concentrations of the flavone isoorientin, an important antioxidant implicated in the intracellular defence mechanism of cancer therapy, was identified for the first time in wild species of pigeonpea.

KEYWORDS
Helicoverpa armigera, isoorientin, leaf bioassay, pigeonpea TPC, resistance, wild relatives

1 | INTRODUCTION

Legumes play an indispensable role in the human diet by supplementing vegetable proteins (20–40%) (Maphosa & Jideani, 2016). Pigeonpea is one such highly nutritious food legume with high levels of protein (up to 32%), essential amino acids like methionine, lysine, tryptophan (David, 2014; Mallikarjuna et al., 2012), calcium (6–94 mg/g), manganese (78–113 mg/g), fibre (1.2–8.2%) and low fat (1.6–2.3%) (Nadimpalli et al., 1993; Saxena & Sultana, 2010). Pigeonpea holds the sixth position in global pulse production and the

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second position in Indian pulse production. However, the productivity of pigeonpea is severely constrained by biotic factors such as pod borer, *Helicoverpa armigera (Hubner)*, causing crop losses of around two billion USD/year (Abigail et al., 2020; Shanower & Minja, 1999).

Screening of 10,000 accessions of the worlds' pigeonpea germplasm at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) did not reveal stable host plant resistance (HPR) to *H. armigera* (Srivastava et al., 1990; Upadhyaya et al., 2011). In recent years, wild species of pigeonpea have attracted attention as a source of resistance to biotic and abiotic stresses. Various biochemical markers, such as stilbenes, oxalic acid, and malic acid (Gadge et al., 2015) for resistance, high soluble sugars, low soluble protein, low condensed tannins, and low phenols for susceptibility were identified (Sharma & Manohar, 2009). For instance, high oxalic acid and malic acid concentrations were essential in *H. armigera* resistant bt-chickpea influencing cry1Ac expression. The anti-feedant activity of the acids might interact with the bt-endotoxins, consequently lowering consumption (Surekha & Arjuna, 2013) by the larvae. Macfoy et al. (1983) have reported amino acids and sugars in a susceptible cultivar of cowpea genotype, Vita-1, in higher concentrations than in the resistant variety, emphasizing that the susceptibility could be due to soluble sugars favouring the insect feeding. Likewise, the presence of non-glandular trichomes or low density glandular trichomes was identified as morphological markers for tolerance to *H. armigera* (Glas et al., 2012; Romeis et al., 1999; Sharma & Manohar, 2009; Sujana et al., 2012). The non-glandular trichome density was reported to be associated with the oviposition of *H. armigera*, whitefly and spider mites (Asif et al., 2019; Rakha & Ramasamy, 2017) in resistant potato accessions. Rashid et al. (2012) have identified the sticky exudates from the trichomes, such as flavonoids, terpenoids, and alkaloids (Rashid et al., 2012), which produce toxic chemicals that hinder the insect's growth, causing antibiosis. They may also act as a physical barrier preventing the insect–plant interaction (Abigail et al., 2020; Ranger & Hower, 2001). Enzymatic markers such as trypsin proteinase inhibitors (TPIs) (Swathi et al., 2016), amylase inhibitors (AIs) (Gadge et al., 2015; Rathinam et al., 2019) were reported in resistant wild *Cajanus* species. Increased expression of TPIs, AIs, H_2O_2, and polyphenol oxidase (Meitei et al., 2018; Rutwik et al., 2020) was observed in resistant genotypes, causing metamorphosis. Studies on *C. scarabaeoides* revealed protease inhibitors that may act on insect gut proteases, causing impaired digestion and altered amino acid absorption (Abigail et al., 2020; Swathi et al., 2015). Also, protease inhibitors were reported to act on mid gut proteases of *Menduca sexta* larvae in pigeonpea and black gram (Prasad & Padmasree, 2010), resulting in antifeedant activity, ultimately larval mortality. Despite the intensive research and identification of pod borer resistance traits in wild relatives of *Cajanus* species, the introgression of resistance genes from wild relatives into the cultivars has been limited (Sharma & Upadhyaya, 2016).

The role of secondary metabolites as defence molecules in plant–insect interactions is widely accepted. Among them, phenolic compounds such as flavonoids, lignin, and tannins (Sharma & Manohar, 2009) are vital in protecting the plant against herbivory (Rizwana & Ashok, 2007). An inheritance study on *C. acutifolius* showed a higher concentration of flavonols quercetin and rutin, leading to herbivory mortality (Jadhav et al., 2012) in resistant genotypes. A similar study with groundnut noted the elevated levels of flavonoids during infestation by tobacco armyworm *Spodoptera litura* (Mallikarjuna et al., 2004). Endogenous flavonols in apple leaves were reported as an essential defence mechanism against fungus *Venturia inaequalis* (Mayr & Treutter, 1998; Picinelli & Mangas, 1995) while investigating scab symptoms. Condensed tannins often inhibit the digestion in larvae and denature proteins (Sharma & Manohar, 2009). With an unpleasant bitter taste, they also act as repellents to larvae (Pagare et al., 2015). Therefore, tannins contribute to HPR (Kamila, 2016).

Similarly, lignin increases the physical toughness of the plant and hinders herbivory and subsequent survival. Earlier studies have found that chitinase and flavonoid 3_5 hydroxylase genes were upregulated in wild *C. platycarpus* (*Benth*.) *Maesen* from the very early stage of *H. armigera* infestation (Rathinam et al., 2019), whereas the same response was observed in cultivated pigeonpea at later stage, suggesting a role of flavonoids as immediate defence molecules in HPR.

Recent contributions to the role of phenolic compounds, especially flavonoids, in insect–plant interactions have inspired us to focus on biochemical bases of wild *Cajanus* species for pod borer resistance.

Australia is one of the centres of diversity of pigeonpea, with 15 out of 32 wild *Cajanus* species with desirable agronomic traits (Khoury et al., 2015). Although traits such as heat and drought tolerance have been reported, there has been limited information on the mechanisms underpinning the resistance to major pest *Helicoverpa armigera* in the Australian wild pigeonpea accessions. This article reports on the screening of native Australian wild pigeonpea species for resistance to *H. armigera* and identifies major biochemical mechanisms underpinning the resistance. The study focuses on phenolic compounds in addition to total phenolic content (TPC).

2 | MATERIALS AND METHODS

2.1 | Plant material

The pigeonpea accessions included in this study were obtained from the Australian Grains Gene Bank, Horsham, Victoria. Two experiments were conducted using different sets of wild and domesticated accessions to explore the resistance components. Exp-1 conducted in 2018 used *C. acutifolius* and its interspecific hybrids (ISH) to screen for *H. armigera* resistance. The results of Exp-1 were verified with the second set with a broader range of wild and domesticated accessions in Exp-2 conducted in 2019.

Exp-1 consisted of 22 genotypes (Table 1) comprising five wild *C. acutifolius* (two plants from each of AGG316925WCAJ1 and AGG318215WCAJ1 accessions and one plant from AGG317765WCAJ1 accession. Fifteen interspecific hybrids,

TABLE 1 Identity of genotypes screened for resistance against *Helicoverpa armigera* and their breeding background

S #	AGG#	Breeding stage/name	Pedigree	Genotypes	Year of experiment
1	AGG316925WCAJ1	Wild	*Cajanus acutifolius*	2	2018
2	AGG317765WCAJ1	Wild	*Cajanus acutifolius*	1	2018
3	AGG318215WCAJ1	Wild	*Cajanus acutifolius*	2	2018
4	AGG322862	F1	310,443 (*C. cajan*) × 316,916 (*C. acutifolius*)	3	2018
5	AGG323314	BC1 W	310,447 (*C. hybrid*) × 316,916 (*C. acutifolius*)	3	2018
6	AGG323318	BC1 W	322,862 (*C. hybrid*) × 316,916 (*C. acutifolius*)	3	2018
7	AGG323331	BC1 D	310,447 (C. hybrid) × 316,916 (*C. cajan*)	3	2018
8	AGG323215	BC1 D	310,447 (C. hybrid) × 316,916 (*C. cajan*)	3	2018
9	ICPL14425	Domesticated	*Cajanus cajan*	2	2018
10	AGG300129WCAJ1	*Cajanus lanceolatus* (W. Fitzg.) Maesen	Wild	1	2019
11	AGG300159WCAJ1	*Cajanus reticulatus.var.reticulatus* (Dryand.) F.Muell.	Wild	1	2019
12	AGG300161WCAJ1	*Cajanus reticulatus* (Dryand.) F. Muell.	Wild	1	2019
13	AGG300162WCAJ1	*Cajanus reticulatus* (Dryand.) F. Muell.	Wild	1	2019
14	AGG309206WCAJ1	*Cajanus pubescens* (Ewart & Morrison) Maesen	Wild	1	2019
15	AGG309207WCAJ1	*Cajanus latisepalus* Maesen	Wild	1	2019
16	AGG309208WCAJ1	*Cajanus latisepalus* Maesen	Wild	1	2019
17	AGG316914WCAJ1	*Cajanus acutifolius* (F. Muell.) Maesen	Wild	1	2019
18	AGG316926WCAJ1	*Cajanus lanuginosus* (S. T. Reynolds & Pedley) Maesen	Wild	1	2019
19	AGG316931WCAJ1	*Cajanus lanuginosus* (S. T. Reynolds & Pedley) Maesen	Wild	1	2019
20	AGG317718WCAJ2	*Cajanus scarabaeoides* (L.) Thouars	Wild	1	2019
21	AGG317719WCAJ3	*Cajanus scarabaeoides* (L.) Thouars	Wild	1	2019
22	AGG310433WCAJ2	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019
23	AGG310443WCAJ2	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019
24	AGG310447WCAJ2	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019
25	ICPL14425	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019
26	QUEST	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019
27	ICPV86022	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019
28	ICPV88039	*Cajanus cajan* (L.) Millsp.	Domesticated	1	2019

Note: (AGG#: Australian grains Genebank number) *BC W; genotypes backcrossed with wild parent, BC D; genotypes backcrossed with parent domesticated, -1,-2,-3 plant number of the same plot.

containing three F1's of *C. acutifolius*, six genotypes backcrossed with wild parent (BC1W), six genotypes backcrossed with domesticated parent (BC1D) and two domesticated genotypes.

Exp-2 consisted of 19 genotypes, including 12 wild accessions, representing seven Australian wild *Cajanus* species and seven domesticated accessions (Table 1). The 12 wild accessions included two from each of *C. lanuginosus*, *C. latisepalus*, *C. reticulatus*, *C. scarabaeoides*, and a single accession each from *C. acutifolius*, *C. lanceolatus*, *C. pubescence*, *C. reticulatus var. reticulatus*., *C. lanuginosus*, is the only species that belonged to the tertiary genepool, whereas the rest of the seven wild accessions belonged to the secondary genepool. The domesticated genotypes included three accessions (AGG310433WCAJ2, AGG310443WCAJ2, and AGG310447WCAJ2) obtained from Genebank ICPL88039, ICPL86022, ICPL14425, and a UQ pigeonpea cultivar released in 1990 as 'QUEST' from the University of Queensland (Troedson & Meekin, 1990).

The protocol for germination of wild seeds involved seed sterilisation, nicking, and growing at 35°C for 8 h, 25°C for 16 h followed by 14 h of light (Vanambathina et al., 2019) in a contained glasshouse. Both the experiments aimed at screening for resistance to *H. armigera* using a detached leaf bioassay (Rathinam et al., 2019; Sharma et al., 2005). The assays were conducted in the same glasshouse conditions. The samples for the phytochemical analysis were collected at the time of experiment.

2.2 | Leaf bioassay

The original culture of wild *H. armigera* was purchased from a commercial organization (AgBitech, Toowoomba, Queensland, Australia). The insects were reared on a soya and wheat flour diet at room temperature (25–27 °C), with humidity of 65 ± 5%, and 12 h photoperiod until the larvae reached the third instar.

The detached leaf bioassay protocol consisted of insect rearing jars with firmly holding ventilated lids (Figure 1). A bed of wet sand (2–3 cm depth) was laid out, and two to three young trifoliate leaves with petioles were inserted into the damp sand vertically in the jars. The third instar larvae's initial weight (WD1) was measured and placed on the leaf at one larva/jar (Figure 1b). Each genotype was tested in three independent jars (replicates). Jars were randomised were kept closed using ventilated lids. Herbivory growth was measured every alternate day by weight, and the fresh leaf material was provided as needed. The jars were re-randomised once 2 days to minimise any local effects. This procedure was continued for 7 days. The herbivory weight difference (HWD) between the initial (WD1) and final weight (WD7) (Figure 1e) served as an indicator for antibiosis (Figure 1f), which was used to rank the genotypes for their level of resistance (Figure 1g). The leaf treatments (genotypes) on which the larvae could not survive when feeding on the leaves were identified as resistant. In contrast, the treatments on which the herbivory could survive by consuming the leaf but failed to pupate were considered medium resistant. Treatments in which herbivory survival was 100%, and the herbivory growth rates were high were reported as susceptible hosts (Brooks, 2008; Vawdrey & De Faveri, 2005).

2.3 | Sample preparation for phytochemical analysis

Flowers were collected from genotypes in Exp-1 and fully opened third and fourth leaves from the secondary branches of genotypes in Exp-2 for phytochemical analysis. All samples were freeze dried for 48 h and then pulverized in a retsch_MM400 ball mill (Retsch-Allee 1–5, 42,781 Haan, Germany) for 30 seconds at 25 oscillations speed. The fine powder was stored in a labelled, airtight container (Liu, Kong, et al., 2010) at −20°C for further analysis. Polyphenol standards (HPLC grade), including apigenin, chlorogenic acid, luteolin, orientin, quercetin, rutin-trihydrate, and vitexin, were sourced from Sigma

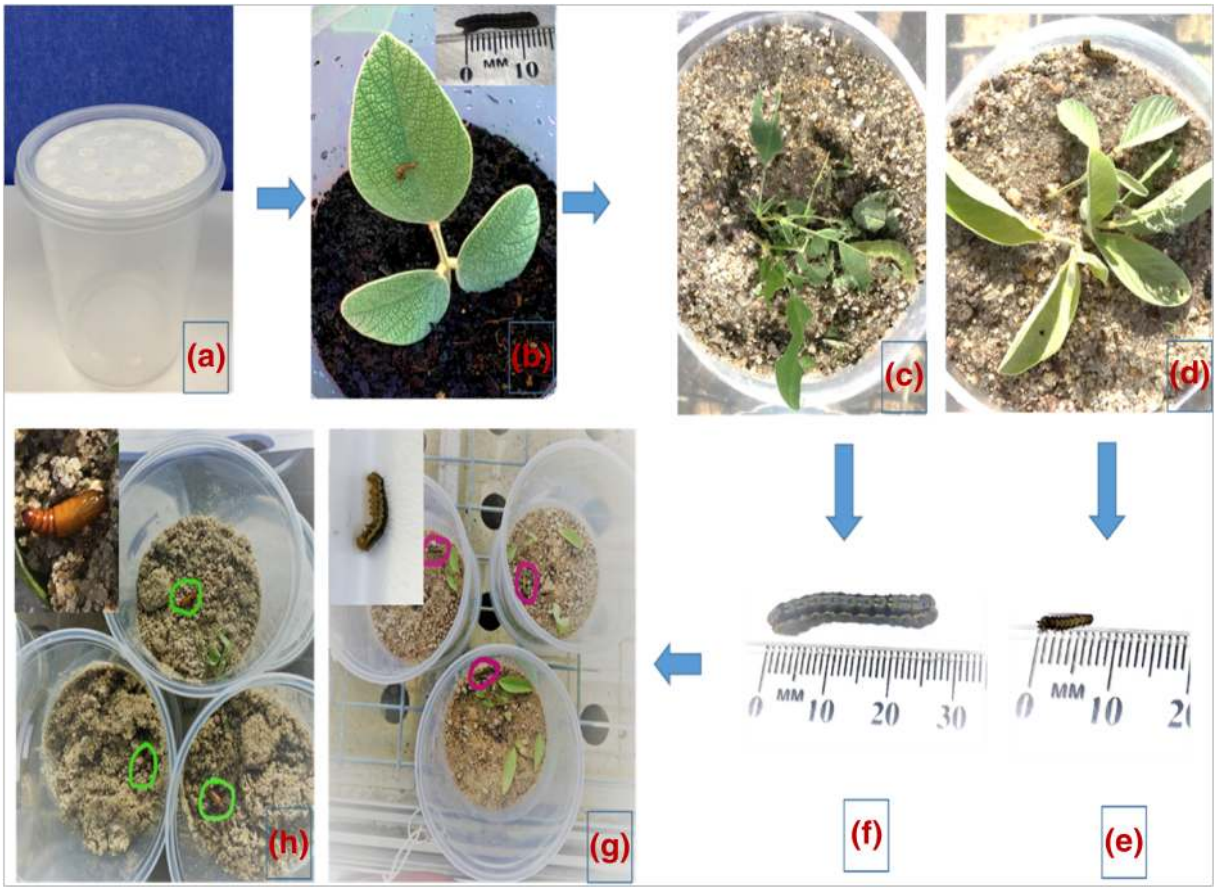


FIGURE 1 (a) Customised jar for the leaf bioassay, (b) third instar larvae of *Helicoverpa armigera* feeding on the leaf, (c) highly consumed susceptible genotype and the healthy larvae (green among the leaves), (d) resistant genotype and the reluctant larvae (black at the sides), (e) dead larvae, (f) healthy larvae, (g) dead larvae in all the three reps (resistant genotype) and (h) larvae pupated in all the three replications (susceptible genotype)

Aldrich (Castle Hill, NSW, Australia). Longistylin-C was sourced from ChemFaces (Wuhan, *Hubei*, China). Polyphenol standards were prepared in milliQ water (apigenin, chlorogenic acid, luteolin, quercetin, and rutin-trihydrate), 1 N NaOH (orientin), methanol (longistylin C), and dimethyl sulfoxide (vitexin). HPLC grade methanol, dimethyl sulfoxide, formic acid, and folin–ciocalteu reagents were sourced from Sigma Aldrich (Castle Hill, NSW, Australia).

2.4 | Extraction of phenolic compounds

Polyphenols were extracted as per the already reported method (Liu, Kong, et al., 2010) with slight modifications. Approximately 100 mg of the powdered material was macerated with 80% methanol containing 1% HCl at room temperature overnight. Next, tubes were incubated in a sonication bath at room temperature for 30 min, followed by centrifugation at 3000 rpm for 5 min (Beckman Coulter Microfuge® 16. Indiana US). The supernatant was collected, whereas the pellet was re-extracted three more times. All the supernatants were combined and subjected to UHPLC-PDA-MS analysis. The extraction was conducted in triplicate. All standards and samples prepared for UPLC were filtered through 0.45 μm nylon membrane filters before use.

2.5 | Total phenolic content (TPC)

TPC was estimated by employing a Folin_Ciocalteu assay as reported previously (Singleton & Rossi, 1965), using a microplate absorbance reader (Sunrise, Tecan, Maennedorf, Switzerland). The absorbance was measured at 700 nm. The TPC concentration was expressed as milligrams of gallic acid equivalents per gram of sample dry weight (GAE mg/g DW), based on a standard curve constructed from a serial dilution of gallic acid (from 0 to 105 mg/L).

2.6 | UPLC-PDA and UHPLC-ESI-MS/MS analysis

The methanolic extract of phenolic compounds was analysed by using a Waters Acquity™ UPLC-PDA system (Waters, Milford, MA, USA). The compounds were separated on a UPLC BEH Shield RP18 Column (2.1 mm × 100 mm; 1.7 μm *i.d.*) maintained at 35°C, with 0.1% formic acid in 10% aqueous methanol (v/v) as mobile phase A and 0.1% formic acid in methanol (v/v) as mobile phase B. The injection volume was 2 μl. The phenolic compounds were estimated using external calibration curves of the individual phenolic compounds, including rutin and quercetin. Polyphenol peak identities were performed by using a Thermo high-resolution Q-exertive mass spectrometer equipped with a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific Pty Ltd, Vic, Australia) was employed to search and match the peaks detected based on their mass spectral characteristics and comparison with phenolic standards as well as with available published data (Liu, Kong, et al., 2010).

2.7 | Herbivory on a diet enriched with leaf extract

The resistance contributed by phenolic compounds could be dosage-dependent. Thus a different experiment was conducted to investigate the role of TPC and isoorientin in contributing to resistance. The diet was enriched with leaf extracts isolated from AGG309208WCAJ1, an accession from the WR group with high TPC and high isoorientin concertation. Another accession, ICPL14425 of DS group with low TPC and low isoorientin concentration; 1.00 g of dry leaf material was used to extract the phenolic compounds following the same protocol used to extract the TPC. The solvent methanol was evaporated, and the residue was dissolved in 70% ethanol; 2 ml of soya insect diet was poured evenly into all cells of the insect rearing plates. Five treatments, including leaf extracts of WR (AGG309208WCAJ1), DS (ICPL14425), DS hiked with WR, isoorientin, and control media enriched with 70% ethanol, were tested at three concentrations of 50, 150, and 200 μl in three replications. Third instar larvae reared on the soya media were weighed initially and fed on randomised treatments for 7 days. The difference in larval weight was recorded every alternate day.

2.8 | Statistical analysis

The data from leaf bioassays and the TPC measurements were subjected to Analysis of Variance (ANOVA), and the least significant difference at 95% confidence level using R software (R Core team 2017). The correlation graphs between insect survival and the TPC for each genotype were produced using Microsoft excel (Praveen et al., 2013). The reported data is the mean of the three replications in bioassay experiments and the nine replications in TPC analysis.

3 | RESULTS

Exp-1 demonstrated a significant variation ($P < 0.001$) in the HPR to *H. armigera* indicated by low HWD ranging between 1 and 94 mg in the native *C. acutifolius* and its interspecific hybrids (BC1W). UPLC analysis of these genotypes indicated the absence of already reported flavonoids such as chlorogenic acid, quercetin and rutin in genotypes expressing HPR (Table 2). This situation led us to focus on TPC concertation. The Exp-1 (Table 2) demonstrated a strong negative relationship between TPC levels and insect survival. The TPC concentration of *C. acutifolius* genotypes was estimated to be two times higher than that in domesticated genotypes. Increased TPC levels in wild genotypes used in Exp-1 raised the question about the TPC levels in the wider range of wild species. The Exp-2 was designed with a wider range of accessions of wild and domesticated genotypes. Exp-2 confirmed earlier findings and demonstrated conspicuous variation in HPR in the wild and domesticated accessions. It was apparent that some wild species may not possess higher TPC levels. The 19 accessions of Exp-2 were grouped based on their intrinsic HPR and TPC. The results are discussed below.

3.1 | Leaf bioassay

Based on herbivory weight difference (HWD), the wild genotypes of *C. acutifolius* had expressed a range of resistance (1–93 mg) to *H. armigera*, followed by backcrosses (BC1W) of *C. acutifolius* (8–100 mg). However, a genotype (AGG323318-2) from BC1W expressed higher resistance with an HWD of −24. The larvae fed on the F1s' (AGG322862) and BC1D (AGG323215 and AGG323331), and the domesticated (ICPL14425) genotype showed increased HWD ranging from 114 to 223 mg and pupated successfully suggesting susceptibility. The least significant difference (LSD) at 95% confidence between genotypes and their HWD signifies the resistance by *C. acutifolius* genotypes and its backcrosses (Table 2), separating them from the other genotypes.

Exp-2 had demonstrated significant ($P > 0.005$) variation in HPR between wild and domesticated accessions as well as among wild accessions. Based on the HWD, the accessions were separated into three groups. Six accessions were classified as wild resistant (WR), with a low rate of HWD (−27–124 mg) (Figure 2). The remaining six wild accessions were categorised as wild susceptible (WS), with HWD ranging between 132 and 207 mg. All seven domesticated accessions were (153–300 mg) categorized under domestic susceptible (DS). Among the WR accessions, *C. pubescens* (AGG309206WCAJ1) was found to be significantly more resistant, with reduced HWD (−27 mg) (Table 3), suggesting a higher level of resistance than *C. acutifolious* (AGG316914WCAJ1), which was proven to be more resistant in Exp-1. The larvae could not survive for 36 h on *C. pubescens* (AGG309206WCAJ1) due to very little leaf consumption in all three replications. Even though two accessions, *C. reticulatus* (AGG300162WCAJ1) and *C. latisepalus* (AGG309208WCAJ1), demonstrated medium levels of antibiosis with an increased mean HWD up to104 mg and 124 mg, respectively (Table 3), they were considered as medium resistant due to failure of larvae to pupate. The remaining genotypes from *C. scarabaeoides* (AGG307718 and AGG317719), *C. reticulatus* (AGG300161), *C. lanuginosus* (AGG316926), and *C. latisepalus* (AGG309207) were categorised under the WS group.

TABLE 2 Variation in larval weight observed in samples tested in 2018

Genotype	HWD (mg)	Rutin (mg/g DW)	Quercetin (mg/g DW)	TPC (GAE mg/g DW)
AGG316925WCAJ1-1_W	18^{c}	0.00^{f}	0.00^{b}	15.42^{bcde}
AGG316925WCAJ1-2_W	1^{c}	0.00^{c}	0.00^{b}	19.08^{abc}
AGG317765WCAJ1-1_W	30^{c}	0.00^{f}	0.00^{b}	18.45^{abc}
AGG318215WCAJ1-1_W	93.7^{bc}	0.00^{f}	0.00^{b}	22.08^{a}
AGG318215WCAJ1-2_W	29.7^{c}	0.00^{f}	0.00^{b}	18.51^{abc}
AGG322862-1_F1	120^{abc}	0.58^{def}	2.45^{a}	13.38^{defg}
AGG322862-2_F1	$114b^{c}$	1.42^{b}	2.59^{a}	14.43^{cdef}
AGG322862-3_F1	$158b^{c}$	1.42^{b}	2.59^{a}	12.24^{cdef}
AGG323215-1_BC1 D	212^{a}	0.67^{def}	2.63^{a}	9.03^{ghij}
AGG323215-2_BC1 D	223^{a}	1.38^{bc}	2.60^{a}	11.42^{efghi}
AGG323215-3_BC1 D	215^{a}	0.71^{cde}	1.88^{a}	9.93^{fghij}
AGG323331-1_BC1 D	205^{a}	0.86^{bcde}	2.46^{a}	12.75^{efgh}
AGG323331-2_BC1 D	181^{ab}	0.87^{bcde}	2.47^{a}	6.70^{j}
AGG323331–3_BC1 D	193^{ab}	2.14^{a}	2.55^{a}	8.75^{ghij}
AGG323314-1_BC1 W	27^{c}	1.09^{bcd}	2.39^{a}	20.30^{a}
AGG323314-2_BC1 W	66^{c}	1.04^{bcd}	2.48^{a}	19.35^{ab}
AGG323314-3_BC1 W	8^{c}	0.00^{f}	0.00^{b}	17.60^{abcd}
AGG323318-1_BC1 W	100^{bc}	0.00^{f}	0.00^{b}	25.40a
AGG323318–2_BC1 W	-24^{c}	0.00^{f}	0.00^{b}	24.80a
AGG323318-3_BC1 W	108^{bc}	0.00^{f}	0.00^{b}	27.60a
ICPL14425-1_D	177^{ab}	1.26^{bcd}	2.71^{a}	9.60^{ghij}
ICPL14425-2_D	213^{a}	0.21^{ef}	2.80^{a}	6.92^{ij}

Note: Rutin, quercetin concentrations and total phenolic content (TPC) in flower samples. Each value is the mean of three replicates. Values with the same letter were not significantly different.

Abbreviations: HWD = herbivory weight difference, DW = dry weight, GAE = gallic acid equivalents, TPC = total phenolic content; -1, -2 = plant number of the same genotype, W = wild, BC1D = back cross with domesticated, BC1W = back cross with wild, D = domesticated.

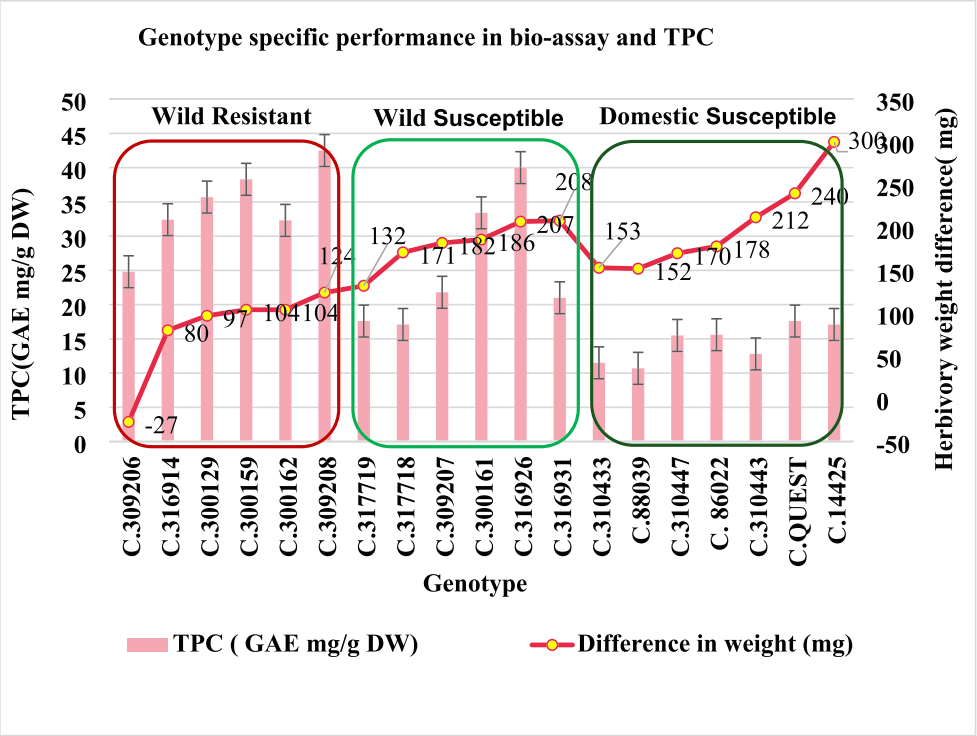


FIGURE 2 Genotypes grouped based on their performance in the detached leaf bioassay and TPC content showing negative correlation of TPC with larval weight gain at $R^2 = 0.713$

TABLE 3 Variation in larval weight (mg) and total phenolic content (GAE mg/g dry weight) estimated in the leaf samples (2019)

Herbivory weight difference and TPC content					
Accession number	**HWD (mg)**	**Group**	**Accession number**	**TPC (GAE mg/g DW)**	**Group**
AGG309206WCAJ1	-27^{c}	WR	AGG300129WCAJ1	35.69^{abc}	W_htpc
AGG316914WCAJ1	80^{abc}	WR	AGG300159WCAJ1	38.32^{ab}	W_htpc
AGG300129WCAJ1	97^{abc}	WR	AGG300161WCAJ1	33.38^{abcd}	W_htpc
AGG300159WCAJ1	104^{abc}	WR	AGG300162WCAJ1	32.41^{abcde}	W_htpc
AGG300162WCAJ1	104^{abc}	WR	AGG309208WCAJ1	42.46^{a}	W_htpc
AGG309208WCAJ1	124^{abc}	WR	AGG316914WCAJ1	32.29^{abced}	W_htpc
AGG317719WCAJ3	132^{abc}	WS	AGG316926WCAJ1	40.20^{a}	W_htpc
AGG317718WCAJ2	171^{ab}	WS	AGG309206WCAJ1	24.77^{abcde}	W_ltpc
AGG309207WCAJ1	182^{ab}	WS	AGG309207WCAJ1	21.77^{abcde}	W_ltpc
AGG300161WCAJ1	186^{ab}	WS	AGG316931WCAJ1	20.94^{abcde}	W_ltpc
AGG316926WCAJ1	207^{a}	WS	AGG317718WCAJ2	17.06^{bcde}	W_ltpc
AGG316931WCAJ1	208^{a}	WS	AGG317719WCAJ3	17.61^{bcde}	W_ltpc
AGG310447WCAJ2	170^{ab}	DS	ICPL14425	17.18^{bcde}	D_ltpc
ICPL14425	178^{ab}	DS	ICPV86022	15.63^{cde}	D_ltpc
AGG310433WCAJ2	200^{a}	DS	ICPV88039	10.66^{e}	D_ltpc
AGG310443WCAJ2	212^{a}	DS	AGG310433WCAJ2	11.52^{de}	D_ltpc
ICPV88039	240^{a}	DS	AGG310443WCAJ2	12.76^{de}	D_ltpc
QUEST	240^{a}	DS	AGG310447WCAJ2	15.48^{cde}	D_ltpc
ICPV86022	245^{a}	DS	QUEST	17.57^{bcde}	D_ltpc
$P > 0.005$**			$P > 0.0002$**		

Note: Values with the same alphabets are not significant.
Abbreviations: HWD = herbivory weight difference TPC = total phenolic content.
**P* value is significant.
***P* value is highly significant.

3.2 | Concentrations of flavonoids

In this study, UPLC-DAD analysis of leaf extracts could not detect apigenin, luteolin, vitexin, and iso-vitexin, the common flavonoids in domesticated pigeonpea. Instead, the spectra indicated the presence of phenolic compounds that could be near isomers. Three compounds eluted at different retention times (RT: 6.8, 7.3, and 9.4) in both wild and domesticated species (Figure S1) had the same spectra. On the other hand, the phenolic compounds eluting at specific RT in different genotypes had different spectra, which adds complexity in understanding the biochemical basis of resistance. Quercetin and rutin were detected in all the samples except for all wild and one BC1W genotype. The concentration of rutin in the F1's was 0.58–1.43 mg/g, followed by 0.21–1.26 mg/g in domesticated and 0.67–2.14 mg/g in BC1D (Table 2). However, BC1W contained 0.00–1.09 mg/g rutin. Higher levels of rutin (2.14 mg/g) were noted in (AGG323331-3) (Table 2) compared with other genotypes. Quercetin was found in higher amounts than rutin ranging from 2.45–2.59 mg/g in F1s, 1.88–2.63 mg/g in BC1D, followed by domesticated (2.71–2.80 mg/g). BC1W had a range of 0.00–2.48 mg/g of quercetin, the lowest concentration observed in all genotypes.

3.3 | TPC variation in genotypes

High variation in the TPC was observed between the genotypes of different progeny levels (Table 2) in Exp-1. Wild genotypes exhibited a variation of 15.4–22.0 GAE mg/g DW TPC, whereas BC1W showed a variation of 17.6–27.6 GAE mg/g DW followed by F1s (12.3–14.4 GAE mg/g DW) in *C. acutifolius*. A low TPC level was noted in the domesticated and BC1D genotypes (6.7–12.7 GAE mg/g DW). The TPC estimated in the 19 accessions of Exp-2 categorised the samples into three groups (Figure 2). Eight wild accessions showed high concentrations of TPC ranging between 32.3 and 42.5 GAE mg/g DW (Table 3), which were grouped under W_htpc (wild high TPC). This group included five accessions from the WR group, excluding *C. pubescens* (AGG309206 WCAJ1) and including *C. reticulatus* (AGG300161 WCAJ1) and *C. lanuginosus* (AGG316926 WCAJ1) from the WS group. Two accessions of *C. scarabaeoides* (AGG317718WCAJ2 and AGG317719WCAJ3) possessing around 17.0 GAE mg/g DW, *C. lanuginosus* (AGG316931WCAJ1), and *C. latisepalus* (AGG309207WCAJ1), accessions with TPC between 20.9 and 21.7 GAE mg/g DW were grouped under W_ltpc (wild low TPC). All the domesticated accessions were grouped as Dom_ltpc (domesticated low TPC), possessing deficient levels of TPC ranging from 10.7 to 17.6 GAE mg/g DW. The overall analysis of the leaf bioassay and the TPC levels showed a high positive relationship between TPC and insect resistance ($R^2 = 0.69$) (Figure 3), with the variation being significant in HWD ($P < 0.005$) and TPC ($P < 0.0002$) (Table 3). The positive association of TPC with HPR enabled us to set the threshold levels of TPC (32.3–42.5 GAE mg/g DW) for resistance. However, two wild accessions, one of each from *C. reticulatus* (AGG300161 WCAJ1) and *C. lanuginosus* (AGG316926 WCAJ1), were found to be susceptible despite having high TPC levels. However, there was a significant difference in the TPC concentration of the 2018 and 2019 analysed samples. Hence flower and leaf samples of two genotypes grown in 2019 were analysed for the TPC to examine the TPC in different tissues of the same genotypes at the same time. The leaf samples of AGG316926WCAJ1 had 40 GAE mg/g DW, TPC, whereas ICPL14425 was noted with 17.1 GAE mg/g DW. Similarly, 26.6 GAE mg/g DW, TPC was noted in the flower samples of AGG316926WCAJ1, whereas 6.60 GAE mg/g DW was observed in ICPL14425.

3.4 | Identification of new flavonoid in pigeonpea profile

UPLC analysis revealed one largest peak observed at 360 nm in all wild Australian species, whereas this peak was detected in lesser concentration in cultivated genotypes. Further literature search and the LC–MS/MS analysis identified a new compound, isoorientin (Figure S2), contributing to the higher levels of TPC in wild species. The molecular weight of isoorientin (negative ionisation method) was 447 with fragment ions 447, 429, 357 [(M – H)-90], 327 [(M – H)-120], 285[(M – H)-162] (Ibrahim et al., 2015).

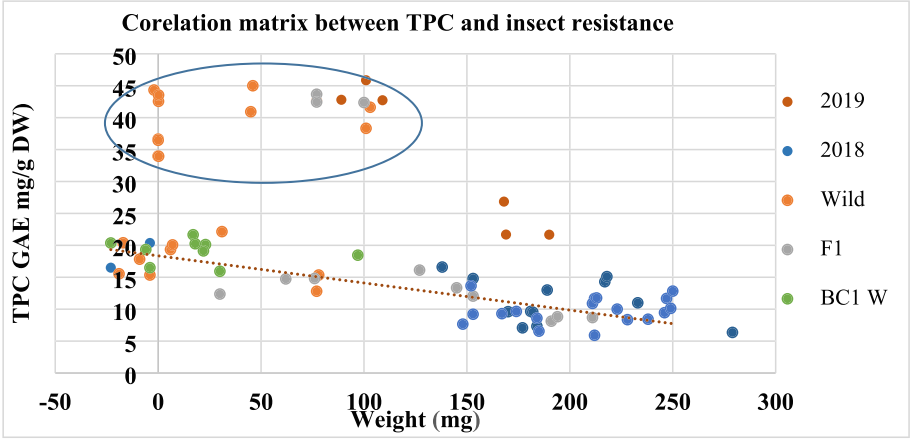


FIGURE 3 The role of total phenolic content (TPC) in the flower and leaf material of genotypic groups used concerning insect survival and their resistance (regression line was drawn using samples analysed in 2018 only, $R^2 = 0.6937$). The points in the circle were not considered for regression, as these are sampled from 2019. However, they still had high TPC and low larval growth

The compound eluted at RT 9.2 in UPLC analysis had the same molecular mass and the fragmented ions, as shown in Figure S2. It was further confirmed by comparison with isoorientin sourced from Sigma Aldrich. Higher concentrations of isoorientin were recorded in all the wild species compared with the domesticated species (Figure 4). However, the role of isoorientin in HPR is yet to be established.

3.5 | Herbivory on a diet enriched with leaf extracts

Herbivory on a diet supplemented with 50 µl of leaf extracts had HWD ranged between 203 and 492 mg in all five treatments (Figure 5). The minimum HWD (203 mg) was observed in the treatment supplemented with WR leaf extract, whereas the remaining four treatments had higher HWD ranging between 395 and 492 mg, indicating their susceptibility. Significantly low HWD (64 mg and 28 mg) were observed in treatments with 150 and 200 µl of WR leaf extracts. A similar pattern was noted in treatments hiked with WR showing low levels of HWD 145 mg and 111 mg in treatments enriched with 150 and 200 µl, respectively. Insects on the remaining three treatments (DS, Isoorientin, and control) enriched with 150 µl were noted to have higher HWD ranging from 286 to 451 mg. However, reduced HWD has observed in the same treatments as the concertation of leaf extracts increased to 200 µl. The decreased HWD with an increase in leaf extracts volume again supports our findings of increased TPC negatively correlates with insect survival.

4 | DISCUSSION

Exploiting wild species to improve genetic diversity and stress mitigation has emerged as a potential approach to crop protection (Mammadov et al., 2018). Despite identifying potential markers for

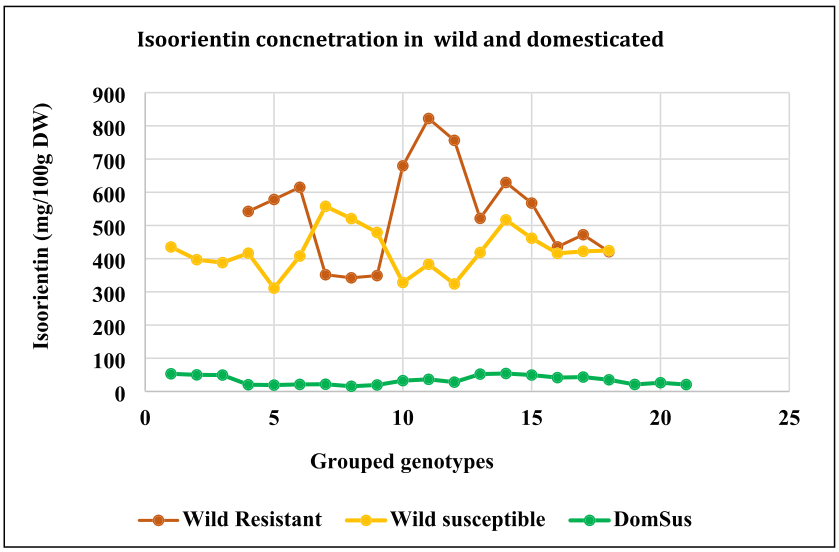


FIGURE 4 Concentration of isoorientin found in three groups of genotypes. The X-axis indicates the total number of genotypes; *y*-axis indicates the concentration of Isoorientin. *mg/100 g DW: Mg of isoorientin in 100 g dry weight of the leaf samples

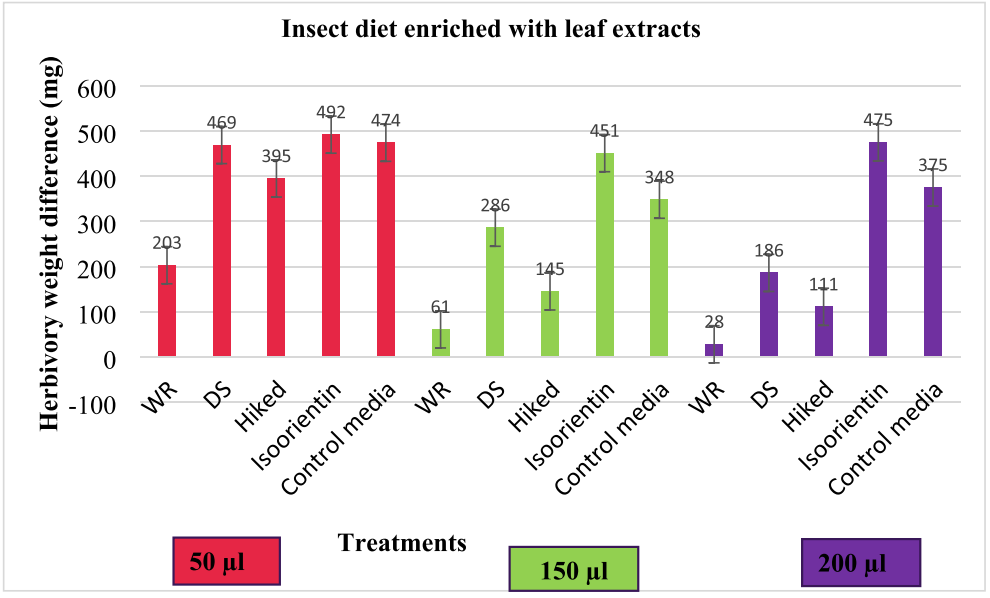


FIGURE 5 Synergic effect of isoorientin in media enriched with leaf extracts experiment. The result is clearly shown by resistant and hiked samples against *H. armigera* third instars in all three treatments. WR, wild resistant; DS, domestic susceptible; hiked, DS hiked with WR leaf extracts

pod borer resistance in wild pigeonpea (Sharma & Manohar, 2009; Swathi et al., 2016), linkage drag has been the major limitation in transferring the resistance through conventional breeding (Venkata et al., 2019). Due to this limitation, attempts were made to develop transgenic pigeonpea with herbivory resistance through transformation using several different approaches, such as pricked embryo axes (*cry1Ac*) (Ajinder et al., 2016), in planta transformation (*cry2Aa* and *cry1AcF*) (Ramkumar et al., 2020) and embryo rescue in pigeonpea (Shivali et al., 2020). Being the second-largest source of *Cajanus* species, Australian wild species have been less exploited for their biotic and abiotic stress-resistance traits. The present investigation of the biochemical basis of resistance has proved the trait potential of Australian native *Cajanus* species. However, the expression of resistance to pod borer varied significantly in the WR group. *C. pubescence* was noted to be highly resistant as larval mortality was observed within 36 h. The remaining five accessions resulted in low HWD before the larval mortality. Lower HWD, increased mortality and prolonged larval development confirms the high (Kumari et al., 2006; Sujana et al., 2008) level of antibiosis existing in native species. A similar kind of resistance was reported in wild species with higher levels of antibiosis and reduced HWD compared with domesticated genotypes in tomatoes (Asif et al., 2019) and chickpea (Sivakumar et al., 2020). On the other hand, wild *Cajanus* species were reported to have traits resistant to *Fusarium* wilt (Mamta et al., 2012; Saxena et al., 2020) and sterility mosaic disease along with other agronomically essential traits like high seed weight and high protein content (Saxena & Rao, 2002; Upadhyaya et al., 2011). Pod borer resistance in BC1W genotypes showed the cross-compatibility and functional integrity of the trait. Hence the members of the WR group would be potential donors for pod borer resistance in pigeonpea.

The UPLC-DAD analysis of leaf extracts and detached leaf bioassay has revealed a significant negative relationship ($R^2 = 0.69$) (Figure 3) between TPC and insect survival. The effect of TPC interfering with the metamorphosis of the insects (Tunaz & Uygun, 2004) was already reported. Phenolic compounds have been targeted as biochemical markers for biotic and abiotic stress resistance since 1986 (Rathi et al., 1986). For instance, the antioxidant activity of *Nypa fruticans*, a palm species, was positively correlated with the TPC concentration in leaves (Hermanto et al., 2020). Increased synthesis of phenylalanine in white cabbage infested by cabbage butterflies and flea beetles was shown to be an induced defence mechanism (Kovalikova et al., 2019). Anket et al. (2016) reported a high TPC concentration, increased expression of chalcone synthase (CHS), and phenylalanine ammonia-lyase (PAL) in stress induced mustard. However, in our study, *C. lanuginosus* (AGG316926WCAJ1) and *C. reticulatus* (AGG300161WCAJ1) were highly susceptible herbivory despite having high TPC. Several other factors, such as high soluble carbohydrates in leaves, might have favoured the herbivory feeding ability (Asif et al., 2019; Sharma & Manohar, 2009), leading to susceptibility.

Moreover, though TPC plays a significant role in plant defence, it has a minimal influence on individual compounds concentration (Pagare et al., 2015). The flower and leaf analysis of AGG316926WCAJ1 and ICPL14425 suggests the concentration of TPC in the leaf was higher than in flowers of the same genotype. Similar results were also reported in *Elaeagnus angustifolia* (Saboonchian & Hosseini, 2014), commonly known as Russian olive had higher phenolic and flavonoid concentration in leaves than in flowers. The higher TPC in resistant accession than in the susceptible accession, suggesting that the TPC level is specific to the genotype. Thus the TPC extracted from any plant tissue of the resistant genotype would be higher than the TPC extracted from the same plant tissue of the susceptible genotype. TPC extracted from different genotypes at the same time should be reliable (Figure 2). Therefore, TPC could be used as a biochemical marker to categorise genotypes resistance to *H. armigera*. TPC and the related enzymes were reported as markers for biotic and abiotic stress in sorghum (Mamoudou et al., 2005). Although our studies indicate the high and low TPC concentration is specific to genotype, Khang and Liu have noted the individual flavonoids could vary depending on tissue and growth stage (Khang et al., 2016; Liu, Zu, et al., 2010). The synthesis of phenols in the chloroplast is a photosensitive phenomenon. Long days in autumn favour increased accumulation of phenolic compounds (Palavan-Unsal, 2011). However, the effect of the environment on TPC accumulation in wild *Cajanus* species is yet to be investigated.

The resistance phenomena observed in the WR group could be a synergistic association of two or more phenolic compounds. Higher expression of quercetin and rutin in pigeonpea (Jadhav et al., 2012), caffeic acid, dihydroxybenzoic acid and vanillic acid in groundnut (Rashid et al., 2016) were reported in *H. armigera* resistance genotypes. Similarly, phenolic compounds extracted from root hairs of tomato tested for *H. armigera*, survival on an artificial diet enriched with leaf extracts reported 53% of larval mortality. This study also showed a high level of antibiosis attributed to the presence of rutin, quercetin, kaempferol, gallic acid and caffeic acid (Harpal et al., 2014). However, the reported resistance was the effect of individual flavonoid or the synergistic effect of TPC with one or more compounds is still unclear. The increased resistance, higher TPC, and isoorientin levels in Australian wild species could be due to the existing genetic difference between Asian and Australian *Cajanus* species (Kassa et al., 2012). However, the basis for the difference in phytochemistry is yet to be identified.

Isoorientin was reported as the most effective compound with great aphidicidal activity on mustard aphid affecting cruciferous vegetables (Gao et al., 2019). The concentration of isoorientin contributing to higher TPC and wild species resistance (Figure 4) could involve a dosage-dependent or a synergistic mechanism. For example, the presence of isoorientin at >0.2% (dry weight) was identified as the concentration required for resistance against *Helicoverpa zea* (Widstrom & Snook, 1998), in conjunction with maysin, in equal concentrations. The hybrids with both compounds were identified as more resistant than those only with isoorientin or maysin (Widstrom & Snook, 1998). Our analysis of herbivory on a diet enriched with leaf extracts (Figure 5) also demonstrated a similar type of mechanism. The herbivory feeding experiments on the WR accession (AGG309208WCAJI) showed a high antibiosis with less HWD than any other treatments. Simultaneously, the feeding on DS spiked with WR caused

continuously increased levels of antibiosis as the concentration of WR leaf extracts increased. In research on tomato, the trichomes extracts were found to contribute significantly to the antibiotic effect of the leaf against *Heliothis zea*. The result was attributed to rutin in synergy with other phenolic compounds (Duffey & Isman, 1981). Our study showed that isoorientin alone might not offer resistance but could act in synergy with other compounds such as maysin in maize (Mamoudou et al., 2005) to contribute to *H. armigera* resistance in the WR group.

5 | CONCLUSION

Our results demonstrated that secondary gene pool of pigeonpea collected in Australia *C. acutifolius* (AGG316925WCAJ1), *C. latisepalus* (AGG309208WCAJ1), *C. lanceolatus* (AGG300129WCAJ1), *C. pubescens* (AGG309206WCAJ1), and *C. reticulatus var. reticulatus* (AGG300159WCAJ1) contain resistance to *H. armigera*. Biochemical analysis of the selected wild accessions and their derivatives revealed that Total Phenolic content (TPCs) could be involved in providing resistance. Limited backcrossing of resistant wild and domesticated susceptible accession indicated that trait could be transferable from the secondary genepool. More importantly, this study indicated variation in phytochemical profiles between domesticated and Australian wild pigeonpea species. Exploring the flavonoid profile of Australian wild species could help to identify the specific compounds acting synergistically. The expression of resistance could also be associated with environmental changes, which requires further investigation.

ETHICS STATEMENT

We declare that the work presented in this paper is the original research work conducted at UQ. It has neither manipulated nor submitted to any other journal before. This paper's authors are not engaged in any personal or financial relationships that can impact this publication.

AUTHOR CONTRIBUTIONS

Prameela Rani Vanambathina carried out the actual laboratory and bioassay experimental work and drafted the manuscript. Rao Rachaputi did the statistical assay assistance and fine changes in manuscript. Yasmina Sultanbawa provided guidance and supervision in phytochemical analysis. Anh Dao Thi Phan assisted in UPLC and LCMS/MS handling and analysis. Robert J. Henry did the manuscript proofreading, grammar, and spell-check. Hugh Brier provided guidance in leaf bioassay and larvae availability.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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