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Pathogenicity Test of Colletotrichum kahawae in Arsi Coffee Growing Areas, Southeastern Ethiopia

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Abstract
Coffee is a non-alcoholic and stimulant beverage crop and belongs to the family Rubiaceae and genus Coffea. Arabica coffee (Coffea arabica L.) has been threatened by various coffee fungal diseases. Among these coffee berry disease (CBD), coffee wilt disease (CWD) and coffee leaf rust (CLR) are the major economically important coffee fungal diseases in Ethiopia. Coffee berry disease (CBD) caused by Colletotrichum kahawae is the major coffee disease in Ethiopia. Experiments were conducted under both laboratory condition. All activities were carried out using completely randomized design (CRD) with three replications. Five representative C. kahawae isolates and one C. gloeosporioides were isolated and identified from infected green coffee berry sampled from the study areas. All isolates showed pathogenic to Arabica coffee. However, there was highly significant variation ($P < 0.05$) among isolates on level of aggressiveness. Isolate Go34 and ShK10 were more highly aggressive to susceptible variety of Arabica coffee ″Arusa″ than the remaining. Any expert can use these virulent isolates in order to generate CBD resistant variety (ies) for the respective areas.
Keywords: Coffea arabica L., Isolates, Colletotrichum kahawae, Pathogenicity

1. Introduction

Pathogenic and pathogenicity variation in *C. kahawae* is usually assessed on hypocotyls or detached berries, and is commonly used as practical implication for disease management. In such tests, small but significant differences are often revealed in the aggressiveness of isolates from different geographic regions (Derso and Waller, 2003) and some authors further suggested the existence of physiological races (Zaffarano, 2009). Unfortunately, these studies were fragmented and sampled *C. kahawae* isolates from different geographic combinations and thus, tended to reach at different conclusions on the most aggressive strains. A more comprehensive study with a broad sampling of fungal isolates is still required.

Tefestewold (1995) studied *C. kahawae* isolates collected from different Ethiopian coffee-growing zones (Hararghe, Ilubabor, Kaffa and Sidama) and demonstrated the variations and similarities in aggressiveness based on pathogenicity tests. A similar study was also conducted by Derso and Waller (2003) in Southwestern Ethiopia (Yirgachefe, Gore and Gera) by collecting *C. kahawae* isolates from garden coffee areas using pathogenicity test on hypocotyls of susceptible coffee cultivars and found isolates that varied in their aggressiveness.

Regarding this aspect at some coffee growing area, related research have been conducted. Likewise, the pathogenicity test of this pathogen has been done at particular coffee growing areas of the country including Borena, Guji, Hararghe, Bale, Jimma, Ilubabor, Kaffa and Sidamo (Tefestewold, 1995; Arega, 2006; Emana, 2014; Abdi and Abu, 2015). According to these authors, all Ethiopian *C. kahawae* isolates/strains showed significant difference in their pathogenicity level on susceptible Arabica coffee varieties. However, in Arsi coffee growing areas, such like research has been limited. Thus, the current study has carried out to characterize/test pathogenicity of the *C. kahawae* isolates collected from different coffee growing areas of Arsi Zone for feature pathology and breeding activities .

2. Materials and Methods

2.1. Description of study site

Study was conducted under laboratory condition in 2017. It was done in Plant Pathology Laboratory of School of Plant Sciences, Haramaya University. Haramaya University was established in 1954 at Haramaya and to be found in Oromia Regional State, Eastern Ethiopia.

2.2. Sample collection and techniques used

Forty-one samples were collected from 41 plots (farms) overall assessed farms during August 2017. From each plot (farm), 40 diseased coffee berries totally, 1640 infected green coffee berries with CBD active lesions were collected from overall assessed sites. These sampled berries were collected and placed into sterilized paper bags and sandwiched between newspapers and kept in a cool box for the pathogen to be viable for successful subsequent isolation. Samples were transported to Plant Pathology Laboratory of School of Plant Sciences, Haramaya University. Samples were maintained at 4^{o}C for further analysis.

2.3. Isolation and identification of *C. kahawae*

Colletotrichum kahawae was isolated from the diseased coffee berries with active CBD lesions using the method

described by Kilimbo *et al*. (2013), Emana (2014), Abdi and Abu (2015) and Fredrick *et al.* (2017). The infected coffee berries with active lesions (sunken and dark lesions) were selected for fungal isolation (Figure 1). The diseased berries were surface-sterilized with 5% sodium hypochlorite (NaOCl) solution for 3 minutes and then rinsed with sterilized distilled water twice for one minute. Sterilized berries were placed on sterilized tissue paper for drying. Totally 30 coffee berries were used and arranged in three replications, i.e. 10 coffee berries per sample were plated on PDA incubated at 25°C for 5 - 7 days.

For the purpose of fungal identification, advanced mycelia was transferred aseptically to freshly prepared potato dextrose agar PDA. The advanced mycelium was taken from the margin of ten-day-old culture by using sterile scalpel. These all activities were done under air-flow laminar hood to reduce contamination arising from airborne micro-organisms (laboratory weeds). Preliminary confirmatory tests of colony texture of *C. kahawae* isolates on PDA was made based on mycological color chart developed by Rayner (1970). Eventually identification of the pathogen was done under compound microscope.

Five representative isolates of *C. kahawae* and one *C. gloeosporioides* were obtained (Table 1). To validate the current findings, isolates that were pathogenic to green coffee berries alone got consideration. Many authors detected *in vitro* testing the on hypocotyls or detached berries (Hindorf *et al*., 1997; Arega, 2006; Nguyen *et al.*, 2010; Kilambo *et al.*, 2013; Abdi and Abu, 2015; Birhanu, 2014). Accordingly, Arsi Zone *C. kahawae* isolates were also examined for their pathogenicity on detached berries collected from susceptible variety.

Those five representative *C. kahawae* isolates were isolated from infected green coffee berries sampled from Chole (one isolate), Gololcha (two isolates) and Shanan Kolu (two isolates), while one *C. gloeosporioides* isolate was obtained from infected green berries samples from Gololcha. In the present study, *C. kahawae* isolates collected from Arsi Zone were characterized for their Morpho-cultural features: mycelium color, mycelial radial growth, mycelia aerial growth rate, conidial production (sporulation capacity), conidial forms/shape, conidial size and their pathogenicity.

Table 3. *C. kahawae* and other *Colletotrichum* spp. isolates detected from infected coffee berries

Isolates code	Garden coffee locality	Pathogenicity	Species
Cho41	Chole	+	*C. Kahawae*
Go33	Gololcha	+	*C. Kahawae*
Go34	Gololcha	+	*C. Kahawae*
Go38	Gololcha	−	*C. gloeosporioides*
Shk9	Shanan Kolu	+	*C. Kahawae*
Shk10	Shanan Kolu	+	*C. Kahawae*

Note: *+ and − stand for pathogenic and non pathogenic of Colletotrichum spp. isolates to coffee berry*

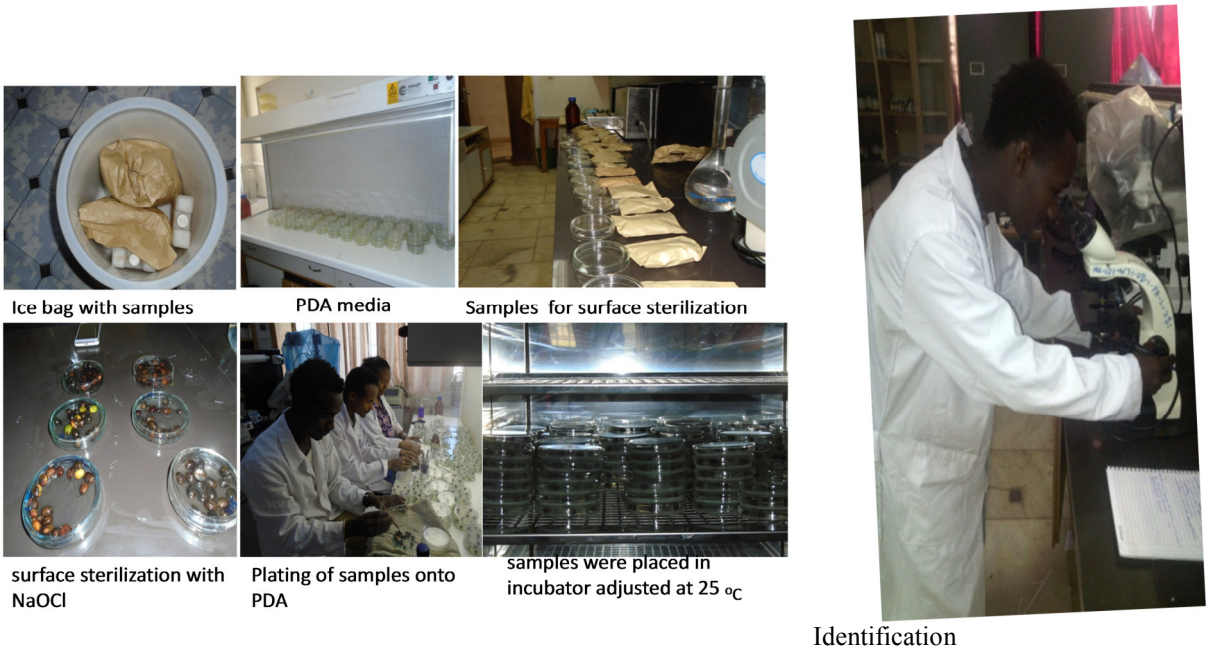

Figure 1. Isolation and identification of *C. kahawae* from infected coffee berry.

2.4. Method and experimental unit used

This test was conducted in Plant Protection Laboratory of Haramaya University using detached berry test (DBT). The pathogenicity of *C. kahawae* isolates were tested on 45 fully expanded, soft green berries 14 weeks old from date of flowering of the susceptible variety "Arusa" maintained at Mechara Agricultural Research Center (McARC). Berries were disinfected using sodium hypochlorite (NaOCl) for 3 minutes and then rinsed with sterilized distilled water twice for one minute (Arega, 2006; Pinard *et al*., 2012; Kilmbo *et al.*, 2013; Emana, 2014; Fredrick *et al.*, 2017).

2.5. Inoculum Preparation C. kahawae Isolates and Inoculation

2.5.1. Inoculum and suspension preparation

Colletotrichum kahawae inoculum was prepared from ten-day-old mono-conidial cultures of each *C. kahawae* isolate incubated on PDA. Advanced mycelia was washed by flooding with 10mL sterile distilled water, rubbed with sterile scalpel and transferred to 50 mL sterilized beaker. To maintain homogeneous and to extract the spores, the suspension was stirred for 10 - 15 minutes by magnetic stirrer. Then the suspension was filtered into another sterile beaker through double layer cheese cloth. Eventually, the prepared conidial suspension was standardized to 2 x 10^6 conidia mL^{-1} by haemocytometer counting, followed by serial dilution (Fredrick *et al.*, 2017).

2.5.2. Inoculation

Fourteen weeks old uninfected coffee berries were collected from susceptible variety "Arusa" maintained at Mechara Agricultural Research Center (McARC). The coffee berries were surface-sterilized with 5% sodium hypochlorite (NaOCl) solution for 3 minutes and then rinsed with sterile distilled water twice for one minute. Sterilized berries were placed on sterile tissue paper for drying. Totally 45 coffee berries were tested from each isolate in three replications, i.e. fifteen berries were tested per replication for each isolate (Figure 2). A drop (25 µL) of the conidia suspension ($2x10^6$ conidia mL^{-1}) was placed at the center of each berry by using sterile pipette (Arega, 2006; Kilimbo *et al*., 2013; Emana, 2014; Fredrick *et al.*, 2017). Then the growth chamber contained inoculated berries was closed after inoculation to maintain the high humidity needed for the infection process and symptom development.

To confirm pathogenicity of the isolates, re-isolation was done following the Koch's postulates. In this case, inoculum was taken from the former tested berries which showed typical symptom of CBD and then plated on PDA medium and followed by sub-culturing, pure colony preparation and its multiplication. Then the 14-day-old healthy immature berries of the susceptible cultivar "Arusa" were inoculated with suspension prepared from the pure colony of each isolates. Finally appearance of symptom was compared with the original one to confirm the pathogenic manner of the isolate(s).

After 14 days, data on index of disease intensity (IDI), which expressed pathogenicity level of each *C. kahawae* isolates were recorded using scales and formula developed by Andreia (2011) (Table 2). Under this active lesion developed by respective *C. kahawae* isolates on each berry

Inoculated healthy coffee berries for pathogenicity test of five Arsi *C. kahawae* isolates

During the pathogenicity data was taken

Figure 3. Inoculated immature coffee berries with five *C. kahawae* isolates for pathogenicity test.

was measured manually in milliliter by using a ruler. Eventually the IDI was computed using the following

formula developed by Andreia (2011).

IDI = ∑ (number of green berries in each class x numeric value of each class)/(total number of green berries x eight)

Table 4. Score scale of disease classification to be used to calculate the index of disease intensity

Class	Description
0	Green berries without symptoms
1	Black points in the inoculation spot (1-2 mm)
2	Black lesions with approximately 3 mm diameter
3	Black lesions with approximately 5 mm diameter
4	Black lesions with approximately 7 mm diameter
5	Black lesions with approximately 10 mm diameter
6	Black lesions with approximately 12 mm diameter
7	Black lesions with approximately 15 mm diameter
8	Whole berries covered with black lesions

Source: Andreia (2011)

2.6. Experimental design, treatments and data analysis:

Completely randomized design (CRD) with three replications was used. Fifteen sterilized berries were arranged in a plastic box lined with wet tissue papers in three replications. Totally forty-five healthy berries were tested for pathogenicity under each isolate. Sterilized distilled water was consistently added or sprayed to berries by using sterilized pipette three times a day (morning, mid-day and evening) to maintain high level of humidity (Kilimbo *et al*., 2013). Fungal growth medium (PDA and MEA) as constant variable and isolates as treatment were used. Data was managed using Excel Spread Data Sheet and finally analyzed using GenStat software version 16 (Duncan's Multiple Range Test).

3. Results and Discussion

3.1. Pathogenicity of Colletotrichum kahawae

All *C. kahawae* isolates were pathogenic to Arabica coffee variety Arusa (a susceptible variety). However, isolates showed significant ($p \leq 0.05$) variation among themselves on their level of aggressiveness or their degree of pathogenicity, i.e. virulence (Table 3). The highest (44.33%) and lowest (22.33%) IDI value was displayed by isolate sampled from Go34 and Shk9, respectively (Table 3). It concluded that Go34 was more aggressive than the remaining isolates, followed by Shk10, Go33, Cho41 and Shk9, whereas Shk9 was less aggressive than the remaining isolates.

As the incubation time increased, particularly above fifteen days, the reaction progressed at decreasing rate along with all isolates and progress in reaction was insignificant beyond 21 days after inoculation. In other words, it indicates that while all isolates were pathogenic to green coffee berry they did not show equal and similar berry invasion ability. Even they showed variation in days to first observable symptom appearance, inducing enlargement of lesions and percent of infected berries. In all, evidence obtained from present study showed that all the five *C. kahawae* isolates were pathogenic to green coffee berries with high variation in virulence.

Isolate Go34 induced CBD symptoms on green coffee berries significantly ($p \leq 0.05$) earlier (at three days after inoculation) than the rest of the isolates. This confirmed the preceding study of Andreia *et al*. (2011) that revealed appearance of first disease CBD symptoms on the green berries inoculated with the isolate Cam1 three days after inoculation. Isolate ShK10 took five days to induce CBD symptoms on green coffee berries. Similarly, isolate Go34 and ShK10 induced significantly larger lesions (mm) than the rest of the isolates, while isolate ShK9 induced the smallest lesions and took nine days to display CBD symptoms.

Isolate Go34 was consistently observed with high virulence to green coffee berries of Arabica coffee variety used in this study. The high virulence ability was recognized to be due to high sporulation capacities, which usually lead to a high percentage of germinated conidia and appresorial formation in the host tissues, resulting high infection levels (Kilimbo *et al*., 2013). The recent study also showed the highest inoculum concentration, leading to highest level of infection (Pinard *et al*., 2012). The previous report compiled by Omondi *et al*. (2001) also indicates presence of virulence variability among *C. kahawae* strains from Ethiopia and Kenya.

In other words, all isolates significantly ($p \leq 0.05$) varied with reference to their aggressiveness on coffee cultivar Arusa compared with a recent study conducted in Kenya (Fredrick *et al*., 2017), where SL28 was highly susceptible with a mean grade of 11.75%. These researchers concluded that variation in *C. kahawae* population was largely due to differences in aggressiveness of the isolates. An earlier finding also revealed that variation of *C. kahawae* population was due to both aggressiveness and some cultural characters, such as rates of sporulation and growth rate (Rodrigues *et al*., 1991).

3.2. Early CBD symptom appearance comparison among isolates

There was highly significant ($p \leq 0.05$) variation among *C. kahawae* isolates related to earlier CBD symptom induction to their host (Table 3). Isolate Go34 germinated within three days post-inoculation compared to the remaining isolates, followed by isolate ShK10, which produced typical symptom of the disease in five days post-inoculation; while isolate ShK9 produced CBD symptom nine days after inoculation. All isolates expressed their infection with different incubation period. In another way, all isolates of *C. kahawae* did not germinate at the same time on a similar host. This indicated that there was variation among isolates with reference to their spore germination on the host. Previous Kilimbo *et al*. (2013) reported that *C. kahawae* isolates induced CBD symptoms on green coffee berries in different days after inoculation as well as they induced varied lesions size (mm).

3.3. Lesion size comparison among isolates

Isolate Go34 produced larger extent lesion size (11.67 mm) than the remaining isolates, followed by isolate ShK10 (10 mm), while isolate ShK9 produced small extent dark sunken lesion size (5 mm), followed by isolate Cho41 (6 mm) (Table 3). According to Kilimbo *et al*. (2013), lesion size appeared on the coffee berry by causal agent of CBD varied from one *C. kahawae* isolate to another.

3.4. Incubation period required to reach maximum invasion (DRTR8-class)

Isolate Go34 was completely destroyed the berry and made mummified berry within 35 days and followed by isolate ShK10 (38 dpi), Go33 (40dpi), Cho41 (42 dpi) and ShK9 (44 dpi) (Table 3). The findings indicated that there was variation between isolates related to their host invasion (colonizing) capacity. In another way, isolate Go34 colonized its berries in short days than the remaining isolates. This showed the existence of active and passive spore germination of isolates with the same fungal species. This variation might be due to existence of genetic diversity among and within *C. kahawae* isolates collected from different agro-ecologies.

Table 5. Aggressiveness, first CBD symptom appearance, lesion size of and days required to reach 8- class (DRTR8-class)

Isolates	Means of aggressiveness	Means of FSA	Means of LS	DRTR8-class
Cho41	23.34c	7ab	6c	42ab
Go33	26.85bc	8a	8b	40abc
Go34	44.33a	3c	11.67a	35c
Shk9	22.25c	9a	5c	44a
Shk10	30.26b	5bc	10a	38bc
f	55.02	10.87	23.25	4.58
P	0.001	0.001	0.001	0.023
LSD	4.017	2.3	1.8	5.146
CV%	8.5	19.8	12.2	7.1

Note: FSA- first CBD symptom appearance, LS- lesion size, DRTR8-class- days required to reach 8-class.

3.5. Correlation Between Virulence and Related Variables

3.5.1. Correlation between pathogenicity and first days to appearance of CBD symptoms

There was positive correlation between pathogenicity (r = 0.164) and first days to appearance of CBD symptom (r= 0.197) (Table 17). Due to earliness in CBD symptom appearance, *C. kahawae* isolates developed larger extended lesion. *C. kahawae* isolate Go34 germinated and expressed black dot CBD symptom within three days post inoculation, followed by isolate ShK10 that produced typical symptom of the pathogen within five days post-inoculation, while isolate ShK9 produced CBD symptom nine days after inoculation. According to Kilimbo *et al*. (2013), *C. kahawae* strains produced large lesion where more virulence than strains characterized with late CBD symptom appearance. Days to first CBD symptom appearance were positively correlated with sporulation capacity (r = 0.53*) Kilimbo *et al*. (2013). A comparable report was presented by Varzea *et al*. (2002) where highly virulent *C. kahawae* strains induced early CBD symptoms.

3.5.2. Correlation between pathogenicity and lesion size

There was positive correlation between pathogenicity (r= 0.164) and lesion size (r = 0.287) (Table 4). Lesion size was positively correlated to sporulation capacity (r = 0.15) (Kilimbo *et al*., 2013). In fact, *C. kahawae* isolate Go34 produced larger (11.67 mm) lesion than the remaining isolates, followed by isolate ShK10 (10 mm), while isolate ShK9 produced small (5 mm) dark sunken lesion, followed by isolate Cho41 (6 mm). Positive correlation was also found between enlargement of lesion size and sporulation capacity of *C. kahawae* isolates studied in Tanzania (Kilimbo *et al*., 2013). According to the author, high percent of berry infection was scored with *C. kahawae* isolates that produced large lesion size.

3.5.3. Correlation between pathogenicity and conidial size

There was negative correlation between pathogenicity (r = 0.164) and lesion size (r = -0.43) (Table 4). Conidial size was positively correlated to sporulation capacity (r = 0.15) (Kilimbo *et al*., 2013). The result of this study was dissimilar to the previous study conducted in Tanzania by Kilimbo *et al*. (2013). According to him, conidial size and virulence of *C. kahawae* were positively correlated (r = -0.087) and were not significantly ($p \leq 0.05$) different, while report made earlier by Hindorf (1970), and Talhinhas *et al*. (2005) demonstrated that spore sizes have no implications in the virulence of *Colletotrichum* species on a host plant. In fact and in case of conidial size, there was high variability within and among Arsi Zone *C. kahawae* isolates. A similar work by Arega (2006) also demonstrated variability in *C. kahawae* isolates sampled from different Ethiopian forest coffee. According to him, there was variable conidial size among and within the pathogen isolates. In another way, since there was high variation between the same isolate of the pathogen on its conidial size, there was insignificant relationship between pathogenicity and conidial size.

3.5.4. Correlation between pathogenicity and sporulation capacity

There was positive correlation between pathogenicity (r = 0.164) and spore production capacity (r = 0.404) (Table 4). Spore production was positively correlated to sporulation capacity (r = 0.15) (Kilimbo *et al*., 2013). Isolates characterized with sporulation capacity infected coffee berries with more severity than isolates which produced lower amount of spore. Isolate Go34 produced large amount of conidia as well as attacked coffee berry more severely than the remaining ones, followed by isolate ShK10, while in contrast isolate ShK9 produced small amount of conidia at the same time attacked coffee berry in less severity, followed by isolate Cho41. A related finding was also made earlier by Varzea *et al.* (2002) when studying virulence variability of *C. kahawae* strains.

Table 6. Correlation between pathogenicity and relative variables under laboratory conditions

Variables	PT	FSA	LS	CS	SC
I	0.164	0.197	0.287	-0.43	0.404
PT	1	-0.911*	0.917*	-0.617	0.897*
FSA		1	-.914*	0.787	-0.899*
LS			1	-0.538	0.992**
CS				1	-0.57
SC					1

Note: I- isolate PT- pathogenicity, FSA- first symptom appearance, LS- lesion size, CS- conidial size, SC- sporulation capacity; ** correlation is highly significant at the 0.01 level (2- tailed); * correlation is significant at the 0.05 level (2- tailed)

4. Summary and Conclusion

Pathogenic variation in *C. kahawae* is usually assessed through pathogenicity tests on hypocotyls or detached berries, and is commonly used as practical implication for disease management. It was conducted under laboratory condition, Haramaya University, Plant Protection Laboratory. Activities was carried out using CRD with three replication. Single factor which was susceptible cultivar "Arusa" and a group of isolates as treatments were used. Accordingly, whether the isolates are pathogenic or not to the coffee berry, they were tested on 14 weeks old healthy green coffee berries collected from susceptible coffee variety "Arusa". Additional Koch postulate was also used in order to confirm the pathogenic traits of the isolates.

All *C. kahawae* isolates were pathogenic to Arabica coffee variety "Arusa". However, isolates showed highly significant variation ($P \leq 0.05$) among them on their level of aggressiveness .i.e. virulence. Among these isolate Go34 was highly aggressive than the remaining, while isolate ShK9 was less aggressiveness.

As a conclusion, present study was characterized *C. kahawae* sampled from Arsi coffee growing areas for their level of virulence. Related to that isolate Go34 and ShK10 were identified as virulent *C. kahawae* isolate. Therefore, any expert can use these virulent isolates in order to generate CBD resistant variety (ies) for the respective areas.

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